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(54) Title: ALZHEIMER'S DISEASE SECRETASE, APP SUBSTRATES THEREFOR, AND USES THEREFOR

(57) Abstract: The present invention provides the enzyme and enzymatic procedures for cleaving the  $\beta$  secretase cleavage site of the APP protein and associated nucleic acids, peptides, vectors, cells and isolates and assays. An enzyme that cleaves the  $\alpha$ -secretase site of APP also is provided. The invention further provides a modified APP protein and associated nucleic acids, peptides, vectors, cells, and cell isolates, and assays that are particularly useful for identifying candidate therapeutics for treatment or prevention of Alzheimer's disease.

Dysregulation of intracellular pathways for proteolytic processing may be central to the pathophysiology of AD. In the case of plaque formation, mutations in APP, PS1 or PS2 consistently alter the proteolytic processing of APP so as to enhance formation of A $\beta$  1-42, a form of the A $\beta$  peptide which seems to be particularly amyloidogenic, and thus very important in AD. Different forms of APP range in size from 695-770 amino acids, localize to the cell surface, and have a single C-terminal transmembrane domain. Examples of specific isoforms of APP which are currently known to exist in humans are the 695-amino acid polypeptide described by Kang *et al.* (1987), *Nature* 325: 733-736 which is designated as the "normal" APP; the 751 amino acid polypeptide described by Ponte *et al.* (1988), *Nature* 331: 525-527 (1988) and Tanzi *et al.* (1988), *Nature* 331: 528-530; and the 770 amino acid polypeptide described by Kitaguchi *et al.* (1988), *Nature* 331: 530-532. The A $\beta$  peptide is derived from a region of APP adjacent to and containing a portion of the transmembrane domain. Normally, processing of APP at the  $\alpha$ -secretase site cleaves the midregion of the A $\beta$  sequence adjacent to the membrane and releases the soluble, extracellular domain of APP from the cell surface. This  $\alpha$ -secretase APP processing creates soluble APP-  $\alpha$ , (sAPP $\alpha$ ) which is normal and not thought to contribute to AD.

Pathological processing of APP at the  $\beta$ - and  $\gamma$ -secretase sites, which are located N-terminal and C-terminal to the  $\alpha$ -secretase site, respectively, produces a very different result than processing at the  $\alpha$  site. Sequential processing at the  $\beta$ - and  $\gamma$ -secretase sites releases the A $\beta$  peptide, a peptide possibly very important in AD pathogenesis. Processing at the  $\beta$ - and  $\gamma$ -secretase sites can occur in both the endoplasmic reticulum (in neurons) and in the endosomal/lysosomal pathway after reinternalization of cell surface APP (in all cells). Despite intense efforts, for 10 years or more, to identify the enzymes responsible for processing APP at the  $\beta$  and  $\gamma$  sites, to produce the A $\beta$  peptide, those proteases remained unknown until this disclosure.

embodiment, the two sets are separated by nucleic acids that code for about 196 amino acids. An exemplary polynucleotide comprises the nucleotide sequence in SEQ ID NO. 5. In another particular embodiment, the two sets of nucleotides are separated by nucleic acids that code for about 190 amino acids. An exemplary

5 polynucleotide comprises the nucleotide sequence in SEQ ID NO. 1. Preferably, the first nucleic acid of the first special set of amino acids, that is, the first special nucleic acid, is operably linked to any codon where the nucleic acids of that codon codes for any peptide comprising from 1 to 10,000 amino acid (positions). In one variation, the first special nucleic acid is operably linked to nucleic acid polymers that code for any

10 peptide selected from the group consisting of: any reporter proteins or proteins which facilitate purification. For example, the first special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathione S transferase, Green Fluorescent protein, and ubiquitin. In another variation, the last nucleic acid of the

15 second set of special amino acids, that is, the last special nucleic acid, is operably linked to nucleic acid polymers that code for any peptide comprising any amino acids from 1 to 10,000 amino acids. In still another variation, the last special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: any reporter proteins or proteins which facilitate purification. For

20 example, the last special nucleic acid is operably linked to nucleic acid polymers that code for any peptide selected from the group consisting of: immunoglobulin-heavy chain, maltose binding protein, glutathione S transferase, Green Fluorescent protein, and ubiquitin.

In a related aspect, the invention provides any isolated or purified nucleic acid

25 polynucleotide that codes for a protease capable of cleaving the beta secretase cleavage site of APP that contains two or more sets of special nucleic acids, where the special nucleic acids are separated by nucleic acids that code for about 100 to 300 amino acid positions, where the amino acids in those positions may be any amino acids, where the first set of special nucleic acids consists of the nucleic acids that code

30 for DTG, where the first nucleic acid of the first special set of nucleic acids is the first

comprising from 100 to 170 codons. In a highly preferred embodiment, the last special nucleic acid is operably linked to nucleic acids comprising from 142 to 163 codons. In a particular embodiment, the last special nucleic acid is operably linked to nucleic acids comprising about 142 codons, or about 163 codons, or about 170  
5 codons. In a highly preferred embodiment, the polynucleotide comprises a sequence that is at least 95% identical to aspartyl-protease encoding sequences taught herein. In one variation, the second set of special nucleic acids code for the peptide DSG. In another variation, the first set of nucleic acid polynucleotide is operably linked to a peptide purification tag. For example, the nucleic acid polynucleotide is operably  
10 linked to a peptide purification tag which is six histidine. In still another variation, the first set of special nucleic acids are on one polynucleotide and the second set of special nucleic acids are on a second polynucleotide, where both first and second polynucleotides have at least 50 codons. In one embodiment of this type, both of the polynucleotides are in the same solution. In a related aspect, the invention provides a  
15 vector which contains a polynucleotide as described above, or a cell or cell line which is transformed or transfected with a polynucleotide as described above or with a vector containing such a polynucleotide.

In still another aspect, the invention provides an isolated or purified peptide or protein comprising an amino acid polymer that is a protease capable of cleaving the  
20 beta ( $\beta$ ) secretase cleavage site of APP that contains two or more sets of special amino acids, where the special amino acids are separated by about 100 to 300 amino acid positions, where each amino acid position can be any amino acid, where the first set of special amino acids consists of the peptide DTG, where the first amino acid of the first special set of amino acids is, the first special amino acid, where the second set of  
25 amino acids is selected from the peptide comprising either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, with the proviso that the proteases disclosed in SEQ ID NO. 2 and SEQ ID NO. 4 are not included. In preferred embodiments, the two sets of amino acids are separated by about 125 to 222 amino acid positions or about 150 to 196 amino acids, or about 150-  
30 190 amino acids, or about 150 to 172 amino acids, where in each position it may be

first amino acid of the first special set of amino acids is, the first special amino acid, D, and where the second set of amino acids is either DSG or DTG, where the last amino acid of the second set of special amino acids is the last special amino acid, G, where the first special amino acid is operably linked to amino acids that code for any number of amino acids from zero to 81 amino acid positions where in each position it may be any amino acid. In a preferred embodiment, the first special amino acid is operably linked to a peptide from about 30-77 or about 64 to 77 amino acids positions where each amino acid position may be any amino acid. In a particular embodiment, the first special amino acid is operably linked to a peptide 35, 47, 71, or 77 amino acids. In a very particular embodiment, the first special amino acid is operably linked to 71 amino acids and the first of those 71 amino acids is the amino acid T. For example, the polypeptide comprises a sequence that is at least 95% identical to an aspartyl protease sequence as described herein. In another embodiment, the first special amino acid is operably linked to any number of from 40 to 54 amino acids (positions) where each amino acid position may be any amino acid. In a particular embodiment, the first special amino acid is operably linked to amino acids that code for a peptide of 47 amino acids. In a very particular embodiment, the first special amino acid is operably linked to a 47 amino acid peptide where the first those 47 amino acids is the amino acid E. In another particular embodiment, the first special amino acid is operably linked to the same corresponding peptides from SEQ ID NO. 3 that are 35, 47, 71, or 77 peptides in length, beginning counting with the amino acids on the first special sequence, DTG, towards the N-terminal of SEQ ID NO. 3. In another particular embodiment, the polypeptide comprises a sequence that is at least 95% identical to the same corresponding amino acids in SEQ ID NO. 4, that is, identical to that portion of the sequences in SEQ ID NO. 4, including all the sequences from both the first and or the second special nucleic acids, toward the terminal, through and including 71, 47, 35 amino acids before the first special amino acids. For example, the complete polypeptide comprises the peptide of 71 amino acids, where the first of the amino acid is T and the second is Q.

group consisting of DSG and DTG; and (c) about 100 to 300 amino acids separating the first and second tripeptide sequences, wherein the polypeptide cleaves the beta secretase cleavage site of amyloid protein precursor. In one embodiment, the polypeptide comprises an amino acid sequence depicted in SEQ ID NO: 2 or 4, whereas in another embodiment, the polypeptide comprises an amino acid sequence other than the amino acid sequences set forth in SEQ ID NOs: 2 and 4. Similarly, the invention provides a purified polynucleotide comprising a nucleotide sequence that encodes a polypeptide that cleaves the beta secretase cleavage site of amyloid protein precursor; wherein the polynucleotide includes a strand that hybridizes to one or more of SEQ ID NOs: 3, 5, and 7 under the following hybridization conditions: hybridization overnight at 42°C for 2.5 hours in 6 X SSC/0.1% SDS, followed by washing in 1.0 X SSC at 65°C, 0.1% SDS. In one embodiment, the polypeptide comprises an amino acid sequence depicted in SEQ ID NO: 2 or 4, whereas in another embodiment, the polypeptide comprises an amino acid sequence other than the amino acid sequences set forth in SEQ ID NOs: 2 and 4. Likewise, the invention provides a purified polypeptide having aspartyl protease activity, wherein the polypeptide is encoded by polynucleotides as described in the preceding sentences. The invention also provides a vector or host cell comprising such polynucleotides, and a method of making the polypeptides using the vectors or host cells to recombinantly express the polypeptide.

In yet another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide, said polynucleotide encoding a Hu-Asp polypeptide and having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:

- (a) a nucleotide sequence encoding a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), wherein said Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) polypeptides have the complete amino acid sequence of SEQ ID NO. 2, SEQ ID NO. 4, and SEQ ID NO. 6, respectively; and
- (b) a nucleotide sequence complementary to the nucleotide sequence of (a).

least 95% identical to a sequence comprising the amino acid sequence of SEQ ID NO.

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In still another aspect, the invention provides an isolated antibody that binds specifically to any Hu-Asp polypeptide described herein, especially the polypeptide described in the preceding paragraphs.

The invention also provides several assays involving aspartyl protease enzymes of the invention. For example, the invention provides

a method to identify a cell that can be used to screen for inhibitors of  $\beta$  secretase activity comprising:

- (a) identifying a cell that expresses a protease capable of cleaving APP at the  $\beta$  secretase site, comprising:
  - i) collect the cells or the supernatant from the cells to be identified
  - ii) measure the production of a critical peptide, where the critical peptide is selected from the group consisting of either the APP C-terminal peptide or soluble APP,
  - iii) select the cells which produce the critical peptide.

In one variation, the cells are collected and the critical peptide is the APP C-terminal peptide created as a result of the  $\beta$  secretase cleavage. In another variation, the supernatant is collected and the critical peptide is soluble APP, where the soluble APP has a C-terminus created by  $\beta$  secretase cleavage. In preferred embodiments, the cells contain any of the nucleic acids or polypeptides described above and the cells are shown to cleave the  $\beta$ -secretase site of any peptide having the following peptide structure, P2, P1, P1', P2', where P2 is K or N, where P1 is M or L, where P1' is D, where P2' is A. The method where P2 is K and P1 is M. The method where P2 is N and P1 is L.

In still another aspect, the invention provides novel isoforms of amyloid protein precursor (APP) where the last two carboxy terminus amino acids of that isoform are both lysine residues. In this context, the term "isoform" is defined as any APP polypeptide, including APP variants (including mutations), and APP fragments

e.g., in the presence or absence of a putative modulator of cleavage activity. Thus, in one preferred embodiment, the polypeptide of the invention further includes a marker. For example, the marker comprises a reporter protein amino acid sequence attached to the APP amino acid sequence. Exemplary reporter proteins include a fluorescing protein (e.g., green fluorescing proteins, luciferase) or an enzyme that is used to cleave a substrate to produce a colorimetric cleavage product. Also contemplated are tag sequences which are commonly used as epitopes for quantitative immunoassays.

In a preferred embodiment, the di-lysine-modified APP of the invention is a human APP. For example, human APP isoforms such as APP695, APP751, and APP770, modified to include the two lysines, are contemplated. In a preferred embodiment, the APP isoform comprises at least one variation selected from the group consisting of a Swedish KM-NL mutation and a London V717-F mutation, or any other mutation that has been observed in a subpopulation that is particularly prone to development of Alzheimer's disease. These mutations are recognized as mutations that influence APP processing into A $\beta$ . In a highly preferred embodiment, the APP protein or fragment thereof comprises the APP-Sw  $\beta$ -secretase peptide sequence NLDA, which is associated with increased levels of A $\beta$  processing and therefore is particularly useful in assays relating to Alzheimer's research. More particularly, the APP protein or fragment thereof preferably comprises the APP-Sw  $\beta$ -secretase peptide sequence SEVNLD AEFR (SEQ ID NO: 63).

In one preferred embodiment, the APP protein or fragment thereof further includes an APP transmembrane domain carboxy-terminal to the APP-Sw  $\beta$ -secretase peptide sequence. Polypeptides that include the TM domain are particularly useful in cell-based APP processing assays. In contrast, embodiments lacking the TM domain are useful in cell-free assays of APP processing.

In addition to working with APP from humans and various animal models, researchers in the field of Alzheimer's also have construct chimeric APP polypeptides which include stretches of amino acids from APP of one species (e.g., humans) fused to stretches of APP from one or more other species (e.g., rodent). Thus, in another embodiment of the polypeptide of the invention, the APP protein or fragment thereof



variation, the  $\beta$ -secretase recognition site Y comprises the human APP-Sw  $\beta$ -secretase peptide sequence NLDA. It will be apparent that these preferred variations are not mutually exclusive of each other -- they may be combined in a single polypeptide. The invention further provides a polynucleotide comprising a nucleotide sequence that  
5 encodes such polypeptides, vectors which comprise such polynucleotides, and host cells which comprises such vectors, polynucleotides, and/or polypeptides.

In yet another aspect, the invention provides a method for identifying inhibitors of an enzyme that cleaves the beta secretase cleavable site of APP comprising:

- 10 a) culturing cells in a culture medium under conditions in which the enzyme causes processing of APP and release of amyloid beta-peptide into the medium and causes the accumulation of CTF99 fragments of APP in cell lysates,
- b) exposing the cultured cells to a test compound; and specifically determining whether the test compound inhibits the function of the enzyme by  
15 measuring the amount of amyloid beta-peptide released into the medium and/or the amount of CTF99 fragments of APP in cell lysates;
- c) identifying test compounds diminishing the amount of soluble amyloid beta peptide present in the culture medium and diminution of CTF99 fragments of APP in cell lysates as Asp2 inhibitors. In preferred embodiments, the cultured cells  
20 are a human, rodent or insect cell line. It is also preferred that the human or rodent cell line exhibits  $\beta$  secretase activity in which processing of APP occurs with release of amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates. Among the contemplated test compounds are antisense oligomers directed against the enzyme that exhibits  $\beta$  secretase activity, which oligomers reduce release  
25 of soluble amyloid beta-peptide into the culture medium and accumulation of CTF99 in cell lysates.

In yet another aspect, the invention provides a method for the identification of an agent that decreases the activity of a Hu-Asp polypeptide selected from the group consisting of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b), the method comprising:

fragments or analogs of APP that retain the  $\beta$ -secretase recognition and cleavage site. Thus, in one variation, the substrate polypeptide of the second composition comprises the amino acid sequence SEVNLDAEFR, which includes the  $\beta$ -secretase recognition site of human APP that contains the "Swiss" mutation. In another variation, the

5 substrate polypeptide of the second composition comprises the amino acid sequence EVKMDAEF. In another variation, the second composition comprises a polypeptide having an amino acid sequence of a human amyloid precursor protein (APP). For example, the human amyloid precursor protein is selected from the group consisting of: APP695, APP751, and APP770. Preferably, the human amyloid precursor protein

10 (irrespective of isoform selected) includes at least one mutation selected from a KM-NL Swiss mutation and a V-F London mutation. As explained elsewhere, one preferred embodiment involves a variation wherein the polypeptide having an amino acid sequence of a human APP further comprises an amino acid sequence comprising a marker sequence attached amino-terminal to the amino acid sequence of the human

15 amyloid precursor protein. Preferably, the polypeptide having an amino acid sequence of a human APP further comprises two lysine residues attached to the carboxyl terminus of the amino acid sequence of the human APP. The assays can be performed in a cell free setting, using cell-free enzyme and cell-free substrate, or can be performed in a cell-based assay wherein the second composition comprises a

20 eukaryotic cell that expresses amyloid precursor protein (APP) or a fragment thereof containing a  $\beta$ -secretase cleavage site. Preferably, the APP expressed by the host cell is an APP variant that includes two carboxyl-terminal lysine residues. It will also be appreciated that the  $\beta$ -secretase enzyme can be an enzyme that is expressed on the surface of the same cells.

25 The present invention provides isolated nucleic acid molecules comprising a polynucleotide that codes for a polypeptide selected from the group consisting of human aspartyl proteases. In particular, human aspartyl protease 1 (Hu-Asp1) and two alternative splice variants of human aspartyl protease-2 (Hu-Asp2), a "long" (L) form designated herein as Hu-Asp2(a) and a "short" (S) form designated Hu-Asp2(b).

30 As used herein, all references to "Hu-Asp" should be understood to refer to all of

binds specifically to any of the Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides of the invention are also provided.

The invention also provides a method for the identification of an agent that modulates the activity of any of Hu-Asp-1, Hu-Asp2(a), and Hu-Asp2(b). The inventions  
5 describes methods to test such agents in cell-free assays to which Hu-Asp2 polypeptide is added, as well as methods to test such agents in human or other mammalian cells in which Hu-Asp2 is present.

The invention provides for methods for assaying for human Asp1(hu-Asp1)  $\alpha$ -secretase activity comprising contacting the hu-Asp1 protein with an amyloid precursor  
10 protein (APP) substrate, wherein the substrate contains an  $\alpha$ -secretase cleavage site; and measuring cleavage of the APP substrate at the  $\alpha$ -secretase cleavage site, thereby assaying hu-Asp1  $\alpha$ -secretase activity. An example of  $\alpha$ -secretase activity is APP processing wherein the APP substrate is cleaved at a site adjacent to the cell membrane (at residues Phe<sup>20</sup>Ala<sup>21</sup> in relation to the A $\beta$  peptide). This cleavage results in the release  
15 of a soluble, extracellular domain of APP, known as amyloid alpha peptide (sAPP $\alpha$ ), from the cell surface into the cytoplasm. The sAPP $\alpha$  within the cytoplasm can be detected and quantitated thereby measuring  $\alpha$ -secretase activity.

The hu-Asp1 enzyme used in the methods of the invention can be purified and isolated from a cell which is transfected or transformed with a polynucleotide that  
20 encodes hu-Asp1, such as SEQ ID NO: 1, or a polynucleotide sequence that encodes the the amino acid sequence of SEQ ID NO: 2. Further, the hu-Asp1 protein used in the methods may be a fragment of the amino acid sequence of SEQ ID NO: 2 which retains  $\alpha$ -secretase activity. Possible fragments that may be of use for the methods include those  
25 lacking the transmembrane domain amino acids 469-492 of SEQ ID NO: 2, those fragments that lack the cytoplasmic amino acids 493-492 of SEQ ID NO: 2, those fragments that lack the amino terminal amino acids 1-62 of SEQ ID NO: 2 or combinations thereof.

The invention also encompasses methods of assaying for  $\alpha$ -secretase activity where hu-Asp1 protein and its substrate are brought into contact by a growing cell  
30 transfected or transformed with a polynucleotide encoding the hu-Asp1 protein or a

hu-Asp1 results in an increase in optical density after altering the assay buffer to alkaline pH.

The present invention also provides for methods of assaying for  $\alpha$ -secretase activity comprising contacting hu-Asp1 protein with an APP substrate, determining the level of hu-Asp1  $\alpha$ -secretase activity in the presence and absence of a modulator of hu-Asp1  $\alpha$ -secretase activity and comparing the hu-Asp1 secretase activity in the presence and absence of the modulator. The modulators determined to increase hu-Asp1  $\alpha$ -secretase activity will be identified as candidate Alzheimer's disease therapeutics. The invention also encompasses methods which comprise a step for treating Alzheimer's disease with identified candidate Alzheimer disease therapeutics. The invention also provides for compositions comprising a candidate Alzheimer's disease therapeutic identified by the  $\alpha$ -secretase assaying methods of the invention. Asp1 modulators that reduce Asp1  $\beta$ -secretase activity and increase Asp1  $\alpha$ -secretase activity are highly preferred. Assays for Asp1  $\beta$ -secretase activity are preferred essentially as described in detail herein for Asp2.

The invention provides for Asp1 protease substrate peptides or fragments thereof, wherein said peptides comprise an amino acid sequence consisting of fifty or fewer amino acids which comprise the Asp1 cleavage site having the amino acid sequence GLALALEP. This peptide was derived from the Asp1 amino acid sequence and the discovery of an apparent Asp1 autocatalytic cleavage in acidic conditions. The Asp1 substrate of the invention may also comprise a detectable label, such as a radioactive label, chemiluminescent label, enzymatic label or a fluorescent label. The fluorescently labeled substrate can consist of internally quenched labels as described above.

The invention also encompasses methods comprising the steps of contacting hu-Asp1 protein with an Asp1 substrate under acidic conditions and determining the level of Asp1 proteolytic activity. An example of Asp1 proteolytic activity is the auto-catalytic processing hu-Asp undergoes in acidic environments, wherein cleavage occurs at an amino acid site surrounding Ala<sup>63</sup> and cleaves the amino terminal amino acids of the hu-Asp1 pro-peptide. The hu-Asp1 pro-peptide refers to a secreted form of Asp1 that has completed intercellular processing which resulted in cleavage of its signal sequence.

Another embodiment of the invention is a purified polypeptide comprising a fragment of a hu-Asp1 protein, wherein said polypeptide lacks the hu-Asp1 transmembrane domain and retains hu-Asp1  $\alpha$ -secretase activity. These polypeptides include fragments of hu-Asp1 having the amino acid sequence set forth in SEQ ID NO: 2, and wherein the polypeptide optionally lacks the transmembrane domain amino acids 469-492 of SEQ ID NO: 2, wherein the polypeptide lacks the cytoplasmic domain amino acids 493-518 of SEQ ID NO: 2 as well. In one variation, the invention provides a polypeptide that lacks amino terminal amino acids 1-62 of SEQ ID NO: 2 but retains Asp1 proteolytic activity. Fragments lacking both the aforementioned amino-terminal and carboxy terminal residues are contemplated.

The invention provides for a polypeptide comprising a fragment of hu-Asp1 having the amino acid sequence set forth in SEQ ID NO: 2 and wherein said polypeptide lacks the amino terminal amino acids 1-62 and retains APP processing activity. For example, referring to the Asp1 sequence in SEQ ID NO: 2, this pre-pro portion would correspond to residues 22 to 62. By performing conventional sequence analysis, the corresponding portions of the Asp1 sequence can also be identified.

Another embodiment of the invention is a polypeptide comprising the amino acid sequence at least 95% identical to a fragment of hu-Asp1 protein, wherein said polypeptide and said fragment lack a transmembrane domain and retain hu-Asp1  $\alpha$ -secretase activity. In addition, the invention embodies a polypeptide comprising an amino acid sequence at least 95% identical to a fragment of hu-Asp1 protein, wherein said polypeptide and said fragment lack the amino terminal amino acids corresponding to the pre-pro portion of hu-Asp1 and retain APP processing activity.

Additional features and variations of the invention will be apparent to those skilled in the art from the entirety of this application, including the drawing and detailed description, and all such features are intended as aspects of the invention. Likewise, features of the invention described herein can be re-combined into additional embodiments that are also intended as aspects of the invention, irrespective of whether the combination of features is specifically mentioned above as an aspect or embodiment of the invention. Also, only such limitations which are described herein as critical to the

Sequence ID No. 6: Human Asp-2(b), predicted amino acid sequence. The Asp2(b) amino acid sequence includes a putative signal peptide, pre-propeptide, and propeptide as described above for Asp2(a). The Asp2(b) further includes a transmembrane domain comprising residues 430-452, a cytoplasmic domain  
 5 comprising residues 453-476, and a putative alpha-helical spacer region, comprising residues 395-429, believed to be unnecessary for proteolytic activity, between the protease catalytic domain and the transmembrane domain.

Sequence ID No. 7: Murine Asp-2(a), nucleotide sequence.

Sequence ID No. 8: Murine Asp-2(a), predicted amino acid sequence. The  
 10 proteolytic processing of murine Asp2(a) is believed to be analogous to the processing described above for human Asp2(a). In addition, a variant lacking amino acid residues 190-214 of SEQ ID NO: 8 is specifically contemplated as a murine Asp2(b) polypeptide.

Sequence ID No. 9: Human APP695, nucleotide sequence.

15 Sequence ID No.10: Human APP695, predicted amino acid sequence.

Sequence ID No.11: Human APP695-Sw, nucleotide sequence.

Sequence ID No.12: Human APP695-Sw, predicted amino acid sequence. In the APP695 isoform, the Sw mutation is characterized by a KM-NL alteration at positions 595-596 (compared to normal APP695).

20 Sequence ID No.13: Human APP695-VF, nucleotide sequence.

Sequence ID No.14: Human APP695-VF, predicted amino acid sequence. In the APP 695 isoform, the VF mutation is characterized by a V-F alteration at position 642 (compared to normal APP 695).

Sequence ID No.15: Human APP695-KK, nucleotide sequence.

25 Sequence ID No.16: Human APP695-KK, predicted amino acid sequence.

(APP695 with two carboxy-terminal lysine residues.)

Sequence ID No.17: Human APP695-Sw-KK, nucleotide sequence.

Sequence ID No.18: Human APP695-Sw-KK, predicted amino acid sequence

Sequence ID No.19: Human APP695-VF-KK, nucleotide sequence

30 Sequence ID No.20: Human APP695-VF-KK, predicted amino acid sequence

Sequence ID No. 55: Human APP770 polypeptide sequence. To introduce the KM-NL Swedish mutation, residues KM at positions 670-71 are changed to NL. To introduce the V-F London mutation, the V residue at position 717 is changed to F.

Sequence ID No. 56: Human APP751 encoding polynucleotide sequence.

5      Sequence ID No. 57: Human APP751 polypeptide sequence (Human APP751 isoform).

Sequence ID No. 58: Human APP770-KK encoding polynucleotide sequence.

Sequence ID No. 59: Human APP770-KK polypeptide sequence. (Human APP770 isoform to which two C-terminal lysines have been added).

10      Sequence ID No. 60: Human APP751-KK encoding polynucleotide sequence.

Sequence ID No. 61: Human APP751-KK polypeptide sequence (Human APP751 isoform to which two C-terminal lysines have been added).

Sequence ID Nos. 62-65: Various short peptide sequences described in detail in detailed description.

15      Sequence ID No. 66: Predicted amino acid sequence of human Asp-1ΔTM(His)<sub>6</sub> as described in Example 14.

Sequence ID No. 67: Amino acid sequence of secreted recombinant Asp-1ΔTM(His)<sub>6</sub> as described in Example 14.

20      Sequence ID No. 68: Amino acid sequence of acid-processed form of Asp1Δ(His)<sub>6</sub>.

Sequence ID No. 69: Amino acid sequence of the self-activated acid processing site within Asp-1ΔTM.

Sequence ID No. 70: Amino acid sequence of a peptide that includes the β-secretase processing site within the Swedish mutant form of APP.

25      Sequence ID No. 71: Amino acid sequence of a peptide (residues 17-24) that includes the α-secretase processing site within the Aβ peptide (Aβ<sub>17-28</sub>).

Sequence ID No. 72: Amino acid sequence of a peptide (residues 16-23) that includes the α-secretase processing site within the Aβ peptide (Aβ<sub>16-28</sub>).

Sequence ID No. 73-74: PCR primers described in Example 14.

Figure 12: Figure 11 shows the predicted amino acid sequence (SEQ ID No. 30) of Human-Asp2(a) $\Delta$ TM(His)<sub>6</sub>

### DETAILED DESCRIPTION OF THE INVENTION

5 A few definitions used in this invention follow, most definitions to be used are those that would be used by one ordinarily skilled in the art.

The term " $\beta$  amyloid peptide" means any peptide resulting from beta secretase cleavage of APP. This includes peptides of 39, 40, 41, 42 and 43 amino acids, extending from the  $\beta$ -secretase cleavage site to 39, 40, 41, 42 and 43 amino acids C-terminal to the  
10  $\beta$ -secretase cleavage site.  $\beta$  amyloid peptide also includes sequences 1-6, SEQ ID NOs. 1-6 of US 5,750,349, issued 12 May 1998 (incorporated into this document by reference). A  $\beta$ -secretase cleavage fragment disclosed here is called CTF-99, which extends from  $\beta$ -secretase cleavage site to the carboxy terminus of APP.

When an isoform of APP is discussed then what is meant is any APP  
15 polypeptide, including APP variants (including mutations), and APP fragments that exists in humans such as those described in US 5,766,846, col 7, lines 45-67, incorporated into this document by reference.

The term " $\beta$ -amyloid precursor protein" (APP) as used herein is defined as a polypeptide that is encoded by a gene of the same name localized in humans on the  
20 long arm of chromosome 21 and that includes " $\beta$ AP - here " $\beta$ -amyloid protein" see above, within its carboxyl third. APP is a glycosylated, single-membrane spanning protein expressed in a wide variety of cells in many mammalian tissues. Examples of specific isotypes of APP which are currently known to exist in humans are the 695 amino acid polypeptide described by Kang et. al. (1987) Nature 325:733-736 which is  
25 designated as the "normal" APP (SEQ ID NOs: 9-10); the 751 amino acid polypeptide described by Ponte et al. (1988) Nature 331:525-527 (1988) and Tanzi et al. (1988) Nature 331:528-530 (SEQ ID NOs: 56-57); and the 770-amino acid polypeptide described by Kitaguchi et. al. (1988) Nature 331:530-532 (SEQ ID NOs: 54-55).  
Examples of specific variants of APP include point mutation which can differ in both  
30 position and phenotype (for review of known variant mutation see Hardy (1992)



Caenorhabditis elegans genome. These sequences were then used to identify by homology search Hu-Asp1 and two alternative splice variants of Hu-Asp2, designated herein as Hu-Asp2(a) and Hu-Asp2(b).

5 In a major aspect of the invention disclosed here we provide new information about APP processing. Pathogenic processing of the amyloid precursor protein (APP) via the A $\beta$  pathway requires the sequential action of two proteases referred to as  $\beta$ -secretase and  $\gamma$ -secretase. Cleavage of APP by the  $\beta$ -secretase and  $\gamma$ -secretase generates the N-terminus and C-terminus of the A $\beta$  peptide, respectively. Because over production of the A $\beta$  peptide, particularly the A $\beta_{1-42}$ , has been implicated in the  
10 initiation of Alzheimer's disease, inhibitors of either the  $\beta$ -secretase and/or the  $\gamma$ -secretase have potential in the treatment of Alzheimer's disease. Despite the importance of the  $\beta$ -secretase and  $\gamma$ -secretase in the pathogenic processing of APP, molecular definition of these enzymes has not been accomplished to date. That is, it was not known what enzymes were required for cleavage at either the  $\beta$ -secretase or  
15 the  $\gamma$ -secretase cleavage site. The sites themselves were known because APP was known and the A $\beta_{1-42}$  peptide was known, see US 5,766,846 and US 5,837,672, (incorporated by reference, with the exception to reference to "soluble" peptides). But what enzyme was involved in producing the A $\beta_{1-42}$  peptide was unknown.

Alignment of the amino acid sequences of Hu-Asp2 with other known aspartyl  
20 proteases reveals a similar domain organization. All of the sequences contain a signal sequence followed by a pro-segment and the catalytic domain containing 2 copies of the aspartyl protease active site motif (DTG/DSG) separated by approximately 180 amino acid residues. Comparison of the processing site for proteolytic removal of the pro-segment in the mature forms of pepsin A, pepsin C, cathepsin D, cathepsin E and  
25 renin reveals that the mature forms of these enzymes contain between 31-35 amino acid residues upstream of the first DTG motif. Inspection of this region in the Hu-Asp-2 amino acid sequence indicates a preferred processing site within the sequence GRRIGS as proteolytic processing of pro-protein precursors commonly occurs at site following dibasic amino acid pairs (eg. RR). Also, processing at this  
30 site would yield a mature enzyme with 35 amino acid residues upstream of the first

Hu-Asp2(b). As used herein, all references to "Hu-Asp2" should be understood to refer to both Hu-Asp2(a) and Hu-Asp2(b). Hu-Asp1 is expressed most abundantly in pancreas and prostate tissues, while Hu-Asp2(a) and Hu-Asp2(b) are expressed most abundantly in pancreas and brain tissues. The invention also provides isolated  
5 Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) polypeptides, as well as fragments thereof which exhibit aspartyl protease activity.

The predicted amino acid sequences of Hu-Asp1, Hu-Asp2(a) and Hu-Asp2(b) share significant homology with previously identified mammalian aspartyl proteases such as pepsinogen A, pepsinogen B, cathepsin D, cathepsin E, and renin. P.B.Szecs,  
10 *Scand. J. Clin. Lab. Invest.* 52:(Suppl. 210 5-22 (1992)). These enzymes are characterized by the presence of a duplicated DTG/DSG sequence motif. The Hu-Asp1 and Hu-Asp2 polypeptides disclosed herein also exhibit extremely high homology with the ProSite consensus motif for aspartyl proteases extracted from the SwissProt database.

15 The nucleotide sequence given as residues 1-1554 of SEQ ID NO:1 corresponds to the nucleotide sequence encoding Hu-Asp1, the nucleotide sequence given as residues 1-1503 of SEQ ID NO:3 corresponds to the nucleotide sequence encoding Hu-Asp2(a), and the nucleotide sequence given as residues 1-1428 of SEQ ID NO:5 corresponds to the nucleotide sequence encoding Hu-Asp2(b). The isolation  
20 and sequencing of DNA encoding Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b) is described below in Examples 1 and 2.

As is described in Examples 1 and 2, automated sequencing methods were used to obtain the nucleotide sequence of Hu-Asp1, Hu-Asp2(a), and Hu-Asp2(b). The Hu-Asp nucleotide sequences of the present invention were obtained for both  
25 DNA strands, and are believed to be 100% accurate. However, as is known in the art, nucleotide sequence obtained by such automated methods may contain some errors. Nucleotide sequences determined by automation are typically at least about 90%, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of a given nucleic acid molecule. The actual sequence may be  
30 more precisely determined using manual sequencing methods, which are well known

peptide. Recombinant Asp2(a) expressed in and purified from insect cells was observed to have this amino terminus, presumably as a result of cleavage by a signal peptidase. The sequence ETDEEP represents the amino-terminus of Asp2(a) or Asp2(b) beginning with residue 46 of SEQ ID NO: 4 or 6, as observed when Asp2(a) has been recombinantly produced in CHO cells (presumably after cleavage by both a rodent signal peptidase and another rodent peptidase that removes a propeptide sequence). The Asp2(a) produced in the CHO cells possesses  $\beta$ -secretase activity, as described in greater detail in Examples 11 and 12. Variants and derivatives, including fragments, of Hu-Asp proteins having the native amino acid sequences given in SEQ ID Nos: 2, 4, and 6 that retain any of the biological activities of Hu-Asp are also within the scope of the present invention. Of course, one of ordinary skill in the art will readily be able to determine whether a variant, derivative, or fragment of a Hu-Asp protein displays Hu-Asp activity by subjecting the variant, derivative, or fragment to a standard aspartyl protease assay. Fragments of Hu-Asp within the scope of this invention include those that contain the active site domain containing the amino acid sequence DTG, fragments that contain the active site domain amino acid sequence DSG, fragments containing both the DTG and DSG active site sequences, fragments in which the spacing of the DTG and DSG active site sequences has been lengthened, fragments in which the spacing has been shortened. Also within the scope of the invention are fragments of Hu-Asp in which the transmembrane domain has been removed to allow production of Hu-Asp2 in a soluble form. In another embodiment of the invention, the two halves of Hu-Asp2, each containing a single active site DTG or DSG sequence can be produced independently as recombinant polypeptides, then combined in solution where they reconstitute an active protease.

Thus, the invention provides a purified polypeptide comprising a fragment of a mammalian Asp2 protein, wherein said fragment lacks the Asp2 transmembrane domain of said Asp2 protein, and wherein the polypeptide and the fragment retain the  $\beta$ -secretase activity of said mammalian Asp2 protein. In a preferred embodiment, the purified polypeptide comprises a fragment of a human Asp2 protein that retains the  $\beta$ -

a purified polypeptide comprising an amino acid sequence that includes amino acids 58 to 394 of SEQ ID NO: 4, and that lacks amino acids 22 to 57 of SEQ ID NO: 4;

5 a purified polypeptide comprising an amino acid sequence that includes amino acids 46 to 394 of SEQ ID NO: 4, and that lacks amino acids 22 to 45 of SEQ ID NO: 4; and

a purified polypeptide comprising an amino acid sequence that includes amino acids 22 to 429 of SEQ ID NO: 4.

Also included as part of the invention is a purified polynucleotide comprising a  
10 nucleotide sequence that encodes such polypeptides; a vector comprising a polynucleotide that encodes such polypeptides; and a host cell transformed or transfected with such a polynucleotide or vector.

Hu-Asp variants may be obtained by mutation of native Hu-Asp-encoding nucleotide sequences, for example. A Hu-Asp variant, as referred to herein, is a  
15 polypeptide substantially homologous to a native Hu-Asp polypeptide but which has an amino acid sequence different from that of native Hu-Asp because of one or more deletions, insertions, or substitutions in the amino acid sequence. The variant amino acid or nucleotide sequence is preferably at least about 80% identical, more preferably at least about 90% identical, and most preferably at least about 95%  
20 identical, to a native Hu-Asp sequence. Thus, a variant nucleotide sequence which contains, for example, 5 point mutations for every one hundred nucleotides, as compared to a native Hu-Asp gene, will be 95% identical to the native protein. The percentage of sequence identity, also termed homology, between a native and a variant Hu-Asp sequence may also be determined, for example, by comparing the two  
25 sequences using any of the computer programs commonly employed for this purpose, such as the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wisconsin), which uses the algorithm of Smith and Waterman (*Adv. Appl. Math.* 2: 482-489 (1981)).

Alterations of the native amino acid sequence may be accomplished by any of  
30 a number of known techniques. For example, mutations may be introduced at

Fragments of the Hu-Asp encoding nucleic acid molecules described herein, as well as polynucleotides capable of hybridizing to such nucleic acid molecules may be used as a probe or as primers in a polymerase chain reaction (PCR). Such probes may be used, *e.g.*, to detect the presence of Hu-Asp nucleic acids in *in vitro* assays, as well as in Southern and northern blots. Cell types expressing Hu-Asp may also be identified by the use of such probes. Such procedures are well known, and the skilled artisan will be able to choose a probe of a length suitable to the particular application. For PCR, 5' and 3' primers corresponding to the termini of a desired Hu-Asp nucleic acid molecule are employed to isolate and amplify that sequence using conventional techniques.

Other useful fragments of the Hu-Asp nucleic acid molecules are antisense or sense oligonucleotides comprising a single stranded nucleic acid sequence capable of binding to a target Hu-Asp mRNA (using a sense strand), or Hu-Asp DNA (using an antisense strand) sequence. In a preferred embodiment of the invention these Hu-Asp antisense oligonucleotides reduce Hu-Asp mRNA and consequent production of Hu-Asp polypeptides.

In another aspect, the invention includes Hu-Asp polypeptides with or without associated native pattern glycosylation. Both Hu-Asp1 and Hu-Asp2 have canonical acceptor sites for Asn-linked sugars, with Hu-Asp1 having two of such sites, and Hu-Asp2 having four. Hu-Asp expressed in yeast or mammalian expression systems (discussed below) may be similar to or significantly different from a native Hu-Asp polypeptide in molecular weight and glycosylation pattern. Expression of Hu-Asp in bacterial expression systems will provide non-glycosylated Hu-Asp.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. Hu-Asp polypeptides may be recovered and purified from tissues, cultured cells, or recombinant cell cultures by well-known methods, including ammonium sulfate or ethanol precipitation, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography, and high performance liquid chromatography

Suitable host cells for expression of Hu-Asp polypeptides include prokaryotes, yeast, and higher eukaryotic cells, each of which is discussed below.

The Hu-Asp polypeptides to be expressed in such host cells may also be fusion proteins which include regions from heterologous proteins. Such regions may be included to allow, *e.g.*, secretion, improved stability, or facilitated purification of the polypeptide. For example, a sequence encoding an appropriate signal peptide can be incorporated into expression vectors. A DNA sequence for a signal peptide (secretory leader) may be fused inframe to the Hu-Asp sequence so that Hu-Asp is translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cell promotes extracellular secretion of the Hu-Asp polypeptide. Preferably, the signal sequence will be cleaved from the Hu-Asp polypeptide upon secretion of Hu-Asp from the cell. Nonlimiting examples of signal sequences that can be used in practicing the invention include the yeast Ifactor and the honeybee melatin leader in sf9 insect cells.

In a preferred embodiment, the Hu-Asp polypeptide will be a fusion protein which includes a heterologous region used to facilitate purification of the polypeptide. Many of the available peptides used for such a function allow selective binding of the fusion protein to a binding partner. For example, the Hu-Asp polypeptide may be modified to comprise a peptide to form a fusion protein which specifically binds to a binding partner, or peptide tag. Nonlimiting examples of such peptide tags include the 6-His tag, thioredoxin tag, hemagglutinin tag, GST tag, and OmpA signal sequence tag. As will be understood by one of skill in the art, the binding partner which recognizes and binds to the peptide may be any molecule or compound including metal ions (*e.g.*, metal affinity columns), antibodies, or fragments thereof, and any protein or peptide which binds the peptide, such as the FLAG tag.

Suitable host cells for expression of Hu-Asp polypeptides includes prokaryotes, yeast, and higher eukaryotic cells. Suitable prokaryotic hosts to be used for the expression of Hu-Asp include bacteria of the genera *Escherichia*, *Bacillus*, and *Salmonella*, as well as members of the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. For expression in, *e.g.*, *E. coli*, a Hu-Asp polypeptide may include

vectors, a shuttle vector will also include sequences for replication and selection in *E. coli*. Direct secretion of Hu-Asp polypeptides expressed in yeast hosts may be accomplished by the inclusion of nucleotide sequence encoding the yeast I-factor leader sequence at the 5' end of the Hu-Asp-encoding nucleotide sequence.

5           Insect host cell culture systems may also be used for the expression of Hu-Asp polypeptides. In a preferred embodiment, the Hu-Asp polypeptides of the invention are expressed using an insect cell expression system (see Example 10). Additionally, a baculovirus expression system can be used for expression in insect cells as reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988).

10           In another preferred embodiment, the Hu-Asp polypeptide is expressed in mammalian host cells. Nonlimiting examples of suitable mammalian cell lines include the COS7 line of monkey kidney cells (Gluzman *et al.*, *Cell* 23:175 (1981)), human embryonic kidney cell line 293, and Chinese hamster ovary (CHO) cells. Preferably, Chinese hamster ovary (CHO) cells are used for expression of Hu-Asp  
15           proteins (Example 11).

          The choice of a suitable expression vector for expression of the Hu-Asp polypeptides of the invention will of course depend upon the specific mammalian host cell to be used, and is within the skill of the ordinary artisan. Examples of suitable expression vectors include pcDNA3 (Invitrogen) and pSVL (Pharmacia Biotech). A  
20           preferred vector for expression of Hu-Asp polypeptides is pcDNA3.1-Hygro (Invitrogen). Expression vectors for use in mammalian host cells may include transcriptional and translational control sequences derived from viral genomes. Commonly used promoter sequences and enhancer sequences which may be used in the present invention include, but are not limited to, those derived from human  
25           cytomegalovirus (CMV), Adenovirus 2, Polyoma virus, and Simian virus 40 (SV40). Methods for the construction of mammalian expression vectors are disclosed, for example, in Okayama and Berg (*Mol. Cell. Biol.* 3:280 (1983)); Cosman *et al.* (*Mol. Immunol.* 23:935 (1986)); Cosman *et al.* (*Nature* 312:768 (1984)); EP-A-0367566; and WO 91/18982.

the disease can then be determined by comparing the nucleic acid sequence between affected and unaffected individuals.

In another embodiment, the invention relates to a method of assaying Hu-Asp function, specifically Hu-Asp2 function which involves incubating in solution the  
5 Hu-Asp polypeptide with a suitable substrate including but not limited to a synthetic peptide containing the  $\beta$ -secretase cleavage site of APP, preferably one containing the mutation found in a Swedish kindred with inherited AD in which KM is changed to NL, such peptide comprising the sequence SEVNLDAEFR in an acidic buffering solution, preferably an acidic buffering solution of pH5.5 (see Example 12) using  
10 cleavage of the peptide monitored by high performance liquid chromatography as a measure of Hu-Asp proteolytic activity. Preferred assays for proteolytic activity utilize internally quenched peptide assay substrates. Such suitable substrates include peptides which have attached a paired fluorphore and quencher including but not limited to 7-amino-4-methyl coumarin and dinitrophenol, respectively, such that  
15 cleavage of the peptide by the Hu-Asp results in increased fluorescence due to physical separation of the fluorphore and quencher. Other paired fluorphores and quenchers include bodipy-tetramethylrhodamine and QSY-5 (Molecular Probes, Inc.). In a variant of this assay, biotin or another suitable tag may be placed on one end of the peptide to anchor the peptide to a substrate assay plate and a fluorphore may be  
20 placed at the other end of the peptide. Useful fluorphores include those listed above as well as Europium labels such as W8044 (EG&g Wallac, Inc.). Cleavage of the peptide by Asp2 will release the fluorphore or other tag from the plate, allowing compounds to be assayed for inhibition of Asp2 proteolytic cleavage as shown by an increase in retained fluorescence. Preferred colorimetric assays of Hu-Asp proteolytic  
25 activity utilize other suitable substrates that include the P2 and P1 amino acids comprising the recognition site for cleavage linked to o-nitrophenol through an amide linkage, such that cleavage by the Hu-Asp results in an increase in optical density after altering the assay buffer to alkaline pH.



enhanced green fluorescent protein (EGFP) cDNA in the second cistron. The APP cDNA was modified by adding two lysine codons to the carboxyl terminus of the APP coding sequence. This increases processing of A $\beta$  peptide from human APP by 2-4 fold. This level of A $\beta$  peptide processing is 60 fold higher than is seen in nontransformed HEK293 cells. HEK125.3 cells will be useful for assays of compounds that inhibit A $\beta$  peptide processing. This invention also includes addition of two lysine residues to the C-terminus of other APP isoforms including the 751 and 770 amino acid isoforms, to isoforms of APP having mutations found in human AD including the Swedish KM-NL and V717-F mutations, to C-terminal fragments of APP, such as those beginning with the  $\beta$ -secretase cleavage site, to C-terminal fragments of APP containing the  $\beta$ -secretase cleavage site which have been operably linked to an N-terminal signal peptide for membrane insertion and secretion, and to C-terminal fragments of APP which have been operably linked to an N-terminal signal peptide for membrane insertion and secretion and a reporter sequence including but not limited to green fluorescent protein or alkaline phosphatase, such that  $\beta$ -secretase cleavage releases the reporter protein from the surface of cells expressing the polypeptide.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

#### Example 1

##### Development of a Search Algorithm Useful for the Identification of Aspartyl Proteases, and Identification of *C. elegans* Aspartyl Protease Genes in Wormpep 12

###### *Materials and Methods:*

Classical aspartyl proteases such as pepsin and renin possess a two-domain structure which folds to bring two aspartyl residues into proximity within the active site. These are embedded in the short tripeptide motif DTG, or more rarely, DSG. The DTG or DSG active site motif appears at about residue 25-30 in the enzyme, but at about 65-70 in the proenzyme (prorenin, pepsinogen). This motif appears again about

```

if (pos>0) {
    rest = substr($0,pos+3)      /*get rest of record after first DTG*/
    pos2 = index(rest,"DTG")    /*find second DTG*/
    if (pos2>0) printf ("%s%s\n", ">", $0)    /*report hits*/
5 }
}

```

The AWK script shown above was used to search Wormpep12, which was downloaded from ftp.sanger.ac.uk/pub/databases/wormpep, for sequence entries containing at least two DTG or DSG motifs. Using AWK limited each record to 3000  
10 characters or less. Thus, 35 or so larger records were eliminated manually from Wormpep12 as in any case these were unlikely to encode aspartyl proteases.

#### **Results and Discussion:**

The Wormpep 12 database contains 12,178 entries, although some of these (<10%) represent alternatively spliced transcripts from the same gene. Estimates of  
15 the number of genes encoded in the *C. elegans* genome is on the order of 13,000 genes, so Wormpep12 may be estimated to cover greater than 90% of the *C. elegans* genome.

Eukaryotic aspartyl proteases contain a two-domain structure, probably arising from ancestral gene duplication. Each domain contains the active site motif D(S/T)G  
20 located from 20-25 amino acid residues into each domain. The retroviral (e.g., HIV protease) or retrotransposon proteases are homodimers of subunits which are homologous to a single eukaryotic aspartyl protease domain. An AWK script was used to search the Wormpep12 database for proteins in which the D(S/T)G motif occurred at least twice. This identified >60 proteins with two DTG or DSG motifs.  
25 Visual inspection was used to select proteins in which the position of the aspartyl domains was suggestive of a two-domain structure meeting the criteria described above.

In addition, the PROSITE eukaryotic and viral aspartyl protease active site pattern PS00141 was used to search Wormpep12 for candidate aspartyl proteases.  
30 (Bairoch A., Bucher P., Hofmann K., The PROSITE database: its status in 1997, *Nucleic Acids Res.* 24:217-221(1997)). This generated an overlapping set of Wormpep12 sequences. Of these, seven sequences contained two DTG or DSG

## Example 2

### Identification of Novel Human Aspartyl Proteases Using Database Mining by Genome Bridging

#### 5     **Materials and Methods:**

Computer-assisted analysis of EST databases, cDNA , and predicted polypeptide sequences:

Exhaustive homology searches of EST databases with the CEASP1, F21F8.3, F21F8.4, and F21F8.7 sequences failed to reveal any novel mammalian homologues. TBLASTN searches with R12H7.2 showed homology to cathepsin D, cathepsin E, pepsinogen A, pepsinogen C and renin, particularly around the DTG motif within the active site, but also failed to identify any additional novel mammalian aspartyl proteases. This indicates that the *C. elegans* genome probably contains only a single lysosomal aspartyl protease which in mammals is represented by a gene family that arose through duplication and consequent modification of an ancestral gene.

TBLASTN searches with T18H9.2, the remaining *C. elegans* sequence, identified several ESTs which assembled into a contig encoding a novel human aspartyl protease (Hu-ASP1). As is described above in Example 1, BLASTX search with the Hu-ASP1 contig against SWISS-PROT revealed that the active site motifs in the sequence aligned with the active sites of other aspartyl proteases. Exhaustive, repetitive rounds of BLASTN searches against LifeSeq, LifeSeqFL, and the public EST collections identified 102 EST from multiple cDNA libraries that assembled into a single contig. The 51 sequences in this contig found in public EST collections also have been assembled into a single contig (THC213329) by The Institute for Genome Research (TIGR). The TIGR annotation indicates that they failed to find any hits in the database for the contig. Note that the TIGR contig is the reverse complement of the LifeSeq contig that we assembled. BLASTN search of Hu-ASP1 against the rat and mouse EST sequences in ZooSeq revealed one homologous EST in each database (Incyte clone 700311523 and IMAGE clone 313341, GenBank accession number W10530, respectively).

No.2) near its C-terminus which suggests that the protease is anchored in the membrane. This feature is not found in any other aspartyl protease.

*Cloning of a full-length Hu-Asp-2 cDNAs:*

5           As is described above in Example 1, genome wide scan of the *Caenorhabditis elegans* database WormPep12 for putative aspartyl proteases and subsequent mining of human EST databases revealed a human ortholog to the *C. elegans* gene T18H9.2 referred to as Hu-Asp1. The assembled contig for Hu-Asp1 was used to query for human paralogs using the BLAST search tool in human EST databases and a single  
10       significant match (2696295CE1) with approximately 60% shared identity was found in the LifeSeq FL database. Similar queries of either gb105PubEST or the family of human databases available from TIGR did not identify similar EST clones. cDNA clone 2696295, identified by single pass sequence analysis from a human uterus cDNA library, was obtained from Incyte and completely sequence on both strands.  
15       This clone contained an incomplete 1266 bp open-reading frame that encoded a 422 amino acid polypeptide but lacked an initiator ATG on the 5' end. Inspection of the predicted sequence revealed the presence of the duplicated aspartyl protease active site motif DTG/DSG, separated by 194 amino acid residues. Subsequent queries of later releases of the LifeSeq EST database identified an additional ESTs, sequenced  
20       from a human astrocyte cDNA library (4386993), that appeared to contain additional 5' sequence relative to clone 2696295. Clone 4386993 was obtained from Incyte and completely sequenced on both strands. Comparative analysis of clone 4386993 and clone 2696295 confirmed that clone 4386993 extended the open-reading frame by 31 amino acid residues including two in-frame translation initiation codons. Despite the  
25       presence of the two in-frame ATGs, no in-frame stop codon was observed upstream of the ATG indicating that the 4386993 may not be full-length. Furthermore, alignment of the sequences of clones 2696295 and 4386993 revealed a 75 base pair insertion in clone 2696295 relative to clone 4386993 that results in the insertion of 25 additional amino acid residues in 2696295. The remainder of the Hu-Asp2 coding  
30       sequence was determined by 5' Marathon RACE analysis using a human

human Asp2 sequence followed by DNA sequence analysis gave an additional 980 bp of the coding sequence. The remainder of the 5' sequence of murine Asp-2 was derived from genomic sequence (see below).

5     *Isolation and sequence analysis of the murine Asp-2 gene.*

A murine EST sequence encoding a portion of the murine Asp2 cDNA was identified in the GenBank EST database using the BLAST search tool and the Hu-Asp2 coding sequence as the query. Clone g3160898 displayed 88% shared identity to the human sequence over 352 bp. Oligonucleotide primer pairs specific for  
10     this region of murine Asp2 were then synthesized and used to amplify regions of the murine gene. Murine genomic DNA, derived from strain 129/SvJ, was amplified in the PCR (25 cycles) using various primer sets specific for murine Asp2 and the products analyzed by agarose gel electrophoresis. The primer set Zoo-1 and Zoo-4  
15     amplified a 750 bp fragment that contained approximately 600 bp of intron sequence based on comparison to the known cDNA sequence. This primer set was then used to screen a murine BAC library by PCR, a single genomic clone was isolated and this cloned was confirmed contain the murine Asp2 gene by DNA sequence analysis. Shotgun DNA sequencing of this Asp2 genomic clone and comparison to the cDNA sequences of both Hu-Asp2 and the partial murine cDNA sequences defined the  
20     full-length sequence of murine Asp2 (SEQ ID No. 7). The predicted amino acid sequence of murine Asp2 (SEQ ID No. 8) showed 96.4% shared identity (GCG BestFit algorithm) with 18/501 amino acid residue substitutions compared to the human sequence (Figure 4). The proteolytic processing of murine Asp2(a) is believed to be analogous to the processing described above for human Asp2(a). In addition, a  
25     variant lacking amino acid residues 190-214 of SEQ ID NO: 8 is specifically contemplated as a murine Asp2(b) polypeptide. All forms of murine Asp2(b) gene and protein are intended as aspects of the invention.

examined except thymus and PBLs. Given the relative abundance of Hu-Asp2 transcripts in brain, the regional expression in brain regions was also established. A similar constellation of transcript sizes were detected in all brain regions examined [cerebellum, cerebral cortex, occipital pole, frontal lobe, temporal lobe and putamen] with the highest abundance in the medulla and spinal cord.

### Example 5

#### Northern Blot Detection of HuAsp-1 and HuAsp-2 Transcripts in Human Cell Lines

A variety of human cell lines were tested for their ability to produce Hu-Asp1 and Asp2 mRNA. Human embryonic kidney (HEK-293) cells, African green monkey (Cos-7) cells, Chinese hamster ovary (CHO) cells, HELA cells, and the neuroblastoma cell line IMR-32 were all obtained from the ATCC. Cells were cultured in DME containing 10% FCS except CHO cells which were maintained in  $\alpha$ -MEM/10% FCS at 37 °C in 5% CO<sub>2</sub> until they were near confluence. Washed monolayers of cells ( $3 \times 10^7$ ) were lysed on the dishes and poly A<sup>+</sup> RNA extracted using the Qiagen Oligotex Direct mRNA kit. Samples containing 2  $\mu$ g of poly A<sup>+</sup> RNA from each cell line were fractionated under denaturing conditions (glyoxal-treated), transferred to a solid nylon membrane support by capillary action, and transcripts visualized by hybridization with random-primed labeled (<sup>32</sup>P) coding sequence probes derived from either Hu-Asp1 or Hu-Asp2. Radioactive signals were detected by exposure to X-ray film and by image analysis with a PhosphorImager.

The Hu-Asp1 cDNA probe visualized a similar constellation of transcripts (2.6 kb and 3.5 kb) that were previously detected in human tissues. The relative abundance determined by quantification of the radioactive signal was Cos-7 > HEK 292 = HELA > IMR32.

The Hu-Asp2 cDNA probe also visualized a similar constellation of transcripts compared to tissue (3.0 kb, 4.4 kb, and 8.0 kb) with the following relative abundance; HEK 293 > Cos 7 > IMR32 > HELA.

CGAATTAAATTCCAGCACACTGGCTACTTCTTGTTCTGCATCTCAAAGAAC  
(SEQ ID No. 48) and the flanking primer #275

CGAATTAAATTCCAGCACACTGGCTA (SEQ ID No. 49) to modify the 3' end of  
the APP695 cDNA (SEQ ID No. 15 [nucleotide] and SEQ ID No. 16 [amino acid]).

5 This also added a BstX1 restriction site that will be compatible with the BstX1 site in  
the multiple cloning site of pIRES-EGFP. PCR amplification was performed with a  
Clontech HF Advantage cDNA PCR kit using the polymerase mix and buffers  
supplied by the manufacturer. For "patch" PCR, the patch primer was used at 1/20th  
the molar concentration of the flanking primers. PCR amplification products were  
10 purified using a QIAquick PCR purification kit (Qiagen). After digestion with  
restriction enzymes, products were separated on 0.8% agarose gels and then excised  
DNA fragments were purified using a QIAquick gel extraction kit (Qiagen).

To reassemble a modified APP695-Sw cDNA, the 5' Not1-Bgl2 fragment of  
the APP695-Sw cDNA and the 3' Bgl2-BstX1 APP695 cDNA fragment obtained by  
15 PCR were ligated into pIRES-EGFP plasmid DNA opened at the Not1 and BstX1  
sites. Ligations were performed for 5 minutes at room temperature using a Rapid  
DNA Ligation kit (Boehringer Mannheim) and transformed into Library Efficiency  
DH5a Competent Cells (GibcoBRL Life Technologies). Bacterial colonies were  
screened for inserts by PCR amplification using primers #276 and #275. Plasmid  
20 DNA was purified for mammalian cell transfection using a QIAprep Spin Miniprep  
kit (Qiagen). The construct obtained was designated pMG125.3 (APPSW-KK, SEQ  
ID No. 17 [nucleotide] and SEQ ID No. 18 [amino acid]).

#### Mammalian Cell Transfection:

HEK293 cells for transfection were grown to 80% confluence in Dulbecco's  
25 modified Eagle's medium (DMEM) with 10% fetal bovine serum. Cotransfections  
were performed using LipofectAmine (Gibco-BRL) with 3 µg pMG125.3 DNA and 9  
µg pcDNA3.1 DNA per 10 x 10<sup>6</sup> cells. Three days posttransfection, cells were  
passaged into medium containing G418 at a concentration of 400 µg/ml. After three  
days growth in selective medium, cells were sorted by their fluorescence.

30 Clonal Selection of 125.3 cells by FACS:

0.01M DPBS to avoid non-specific binding. Human A $\beta$  1-40 or 1-42 standards 100  $\mu$ l/well (Bachem, Torrance, CA) diluted, from a 1mg/ml stock solution in DMSO, in culture medium was added after washing the plate, as well as 100  $\mu$ l/well of sample, e.g., conditioned medium of transfected cells.

5           The plate was incubated for 2 hours at room temperature and 4°C overnight. The next day, after washing the plate, 100  $\mu$ l/well biotinylated rabbit antiserum 162 1:400 or 164 1:50 diluted in DPBST + 0.5% BSA was added and incubated at room temperature for 1 hour, 15 minutes. Following washes, 100  $\mu$ l/well neutravidin-horseradish peroxidase (Pierce, Rockford, IL) diluted 1:10,000 in DPBST 10 was applied and incubated for 1 hour at room temperature. After the last washes 100  $\mu$ l/well of o-phenylenediamine dihydrochloride (Sigma Chemicals, St. Louis, MO) in 50mM citric acid/100mM sodium phosphate buffer (Sigma Chemicals, St. Louis, MO), pH 5.0, was added as substrate and the color development was monitored at 15 standards and samples were run in triplicates. The samples with absorbance values falling within the standard curve were extrapolated from the standard curves using Soft max Pro software and expressed in pg/ml culture medium.

**Results:**

20           Addition of two lysine residues to the carboxyl terminus of APP695 greatly increases A $\beta$  processing in HEK293 cells as shown by transient expression (Table 1). Addition of the di-lysine motif to APP695 increases A $\beta$  processing to that seen with the APP695 containing the Swedish mutation. Combining the di-lysine motif with the Swedish mutation further increases processing by an additional 2.8 fold.

25           Cotransformation of HEK293 cells with pMG125.3 and pcDNA3.1 allowed dual selection of transformed cells for G418 resistance and high level expression of EGFP. After clonal selection by FACS, the cell line obtained, produces a remarkable 20,000 pg A $\beta$  peptide per ml of culture medium after growth for 36 hours in 24 well plates. Production of A $\beta$  peptide under various growth conditions is summarized in Table 2.

30



TABLE 2

Release of A $\beta$  peptide from HEK125.3 cells under various growth conditions.

5	Type of Culture	Volume of	Duration of	A $\beta$ 1-40	A $\beta$ 1-42
	Plate	Medium	Culture	(pg/ml)	(pg/ml)
	24 well plate	400 ul	36 hr	28,036	1,439

10

**Example 7****Antisense oligomer inhibition of Abeta processing in HEK125.3 cells**

The sequences of Hu-Asp1 and Hu-Asp2 were provided to Sequitur, Inc (Natick, MA) for selection of targeted sequences and design of 2nd generation chimeric antisense oligomers using proprietary technology (Sequitur Ver. D Pat pending #3002). Antisense oligomers Lot# S644, S645, S646 and S647 were targeted against Asp1. Antisense oligomers Lot# S648, S649, S650 and S651 were targeted against Asp2. Control antisense oligomers Lot# S652, S653, S655, and S674 were targeted against an irrelevant gene and antisense oligomers Lot #S656, S657, S658, and S659 were targeted against a second irrelevant gene.

For transfection with the antisense oligomers, HEK125.3 cells were grown to about 50% confluence in 6 well plates in Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum. A stock solution of oligofectin G (Sequitur Inc., Natick, MA) at 2 mg/ml was diluted to 50  $\mu$ g/ml in serum free MEM. Separately, the antisense oligomer stock solution at 100  $\mu$ M was diluted to 800 nM in Opti-MEM (GIBCO-BRL, Grand Island, NY). The diluted stocks of oligofectin G and antisense oligomer were then mixed at a ratio of 1:1 and incubated at room temperature. After 15 minutes incubation, the reagent was diluted 10 fold into MEM containing 10% fetal calf serum and 2 ml was added to each well of the 6 well plate

TABLE 3

Inhibition of A $\beta$  peptide release from HEK125.3 cells treated with antisense oligomers.

5		Gene Targeted	Antisense Oligomer	Abeta (1-40)	Abeta (1-42)
		Asp1-1	S644	62%*	56%*
		Asp1-2	S645	41%*	38%*
		Asp1-3	S646	52%*	46%*
10		Asp1-4	S647	6%	25%*
		Asp2-1	S648	71%*	67%*
		Asp2-2	S649	83%*	76%*
		Asp2-3	S650	46%*	50%*
		Asp2-4	S651	47%*	46%*
15		Con1-1	S652	13%	18%
		Con1-2	S653	35%	30%
		Con1-3	S655	9%	18%
		Con1-4	S674	29%	18%
		Con2-1	S656	12%	18%
20		Con2-2	S657	16%	19%
		Con2-3	S658	8%	35%
		Con2-4	S659	3%	18%

25 Since HEK293 cells derive from kidney, the experiment was extended to human IMR-32 neuroblastoma cells which express all three APP isoforms and which release A $\beta$  peptides into conditioned medium at measurable levels. [See Neill *et al.*, *J. NeuroSci. Res.*, (1994) 39: 482-93; and Asami-Odaka *et al.*, *Biochem.*, (1995) 34:10272-8.] Essentially identical results were obtained in the neuroblastoma cells as the HEK293 cells. As shown in Table 3B, the pair of Asp2 antisense oligomers

IMR-32 and Neuro-2a cells indicate that Asp2 acts directly or indirectly to facilitate A $\beta$  processing in both somatic and neural cell lines.

#### Example 8

##### 5           **Demonstration of Hu-Asp2 $\beta$ -Secretase Activity in Cultured Cells**

Several mutations in APP associated with early onset Alzheimer's disease have been shown to alter A $\beta$  peptide processing. These flank the -- and C-terminal cleavage sites that release A $\beta$  from APP. These cleavage sites are referred to as the  $\beta$ -secretase and  $\gamma$ -secretase cleavage sites, respectively. Cleavage of APP at the

10        $\beta$ -secretase site creates a C-terminal fragment of APP containing 99 amino acids of 11,145 daltons molecular weight. The Swedish KM-NL mutation immediately upstream of the  $\beta$ -secretase cleavage site causes a general increase in production of both the 1-40 and 1-42 amino acid forms of A $\beta$  peptide. The London VF mutation (V717-F in the APP770 isoform) has little effect on total A $\beta$  peptide production, but

15       appears to preferentially increase the percentage of the longer 1-42 amino acid form of A $\beta$  peptide by affecting the choice of  $\beta$ -secretase cleavage site used during APP processing. Thus, we sought to determine if these mutations altered the amount and type of A $\beta$  peptide produced by cultured cells cotransfected with a construct directing expression of Hu-Asp2.

20           Two experiments were performed which demonstrate Hu-Asp2  $\beta$ -secretase activity in cultured cells. In the first experiment, treatment of HEK125.3 cells with antisense oligomers directed against Hu-Asp2 transcripts as described in Example 7 was found to decrease the amount of the C-terminal fragment of APP created by  $\beta$ -secretase cleavage (CTF99) (Figure 9). This shows that Hu-Asp2 acts directly or

25       indirectly to facilitate  $\beta$ -secretase cleavage. In the second experiment, increased expression of Hu-Asp2 in transfected mouse Neuro2A cells is shown to increase accumulation of the CTF99  $\beta$ -secretase cleavage fragment (Figure 10). This increase is seen most easily when a mutant APP-KK clone containing a C-terminal di-lysine motif is used for transfection. A further increase is seen when Hu-Asp2 is

APP-KK: APP695 containing a C-terminal KK motif (SEQ ID Nos. 15 & 16),

APP-Sw-KK: APP695-Sw containing a C-terminal KK motif (SEQ ID No. 17 & 18),

APP-VF-KK: APP695-VF containing a C-terminal KK motif (SEQ ID Nos. 19 & 20).

These were inserted into the vector pIRES-EGFP (Clontech, Palo Alto CA) between the *NorI* and *BstXI* sites using appropriate linker sequences introduced by PCR.

10 *Transfection of antisense oligomers or plasmid DNA constructs in HEK293 cells, HEK125.3 cells and Neuro-2A cells,*

Human embryonic kidney HEK293 cells and mouse Neuro-2a cells were transfected with expression constructs using the Lipofectamine Plus reagent from Gibco/BRL. Cells were seeded in 24 well tissue culture plates to a density of 70-80% confluence. Four wells per plate were transfected with 2 µg DNA (3:1, APP:cotransfectant), 8 µl Plus reagent, and 4 µl Lipofectamine in OptiMEM. OptiMEM was added to a total volume of 1 ml, distributed 200 µl per well and incubated 3 hours. Care was taken to hold constant the ratios of the two plasmids used for cotransfection as well as the total amount of DNA used in the transfection. The transfection media was replaced with DMEM, 10%FBS, NaPyruvate, with antibiotic/antimycotic and the cells were incubated under normal conditions (37°C, 5% CO<sub>2</sub>) for 48 hours. The conditioned media were removed to polypropylene tubes and stored at -80°C until assayed for the content of Aβ1-40 and Aβ1-42 by EIA as described in the preceding examples. Transfection of antisense oligomers into HEK125.3 cells was as described in Example 7.

25 *Preparation of cell extracts, Western blot protocol*

Cells were harvested after being transfected with plasmid DNA for about 60 hours. First, cells were transferred to 15-ml conical tube from the plate and centrifuged at 1,500 rpm for 5 minutes to remove the medium. The cell pellets were washed once with PBS. We then lysed the cells with lysis buffer (10 mM HEPES, pH

acts directly or indirectly to facilitate the processing and release of A $\beta$  from endogenously expressed APP.

Co-transfection of Hu-Asp2 with APP has little effect on A $\beta$ 40 production but increases A $\beta$ 42 production above background (Table 4). Addition of the di-lysine motif to the C-terminus of APP increases A $\beta$  peptide processing about two fold, although A $\beta$ 40 and A $\beta$ 42 production remain quite low (352 pg/ml and 21 pg/ml, respectively). Cotransfection of Asp2 with APP-KK further increases both A $\beta$ 40 and A $\beta$ 42 production.

The APP V717-F mutation has been shown to increase  $\gamma$ -secretase processing at the A $\beta$ 42 cleavage site. Cotransfection of Hu-Asp2 with the APP-VF or APP-VF-KK constructs increased A $\beta$ 42 production (a two fold increase with APP-VF and a four-fold increase with APP-VF-KK, Table 4), but had mixed effects on A $\beta$ 40 production (a slight decrease with APP-VF, and a two fold increase with APP-VF-KK in comparison to the pcDNA cotransfection control. Thus, the effect of Asp2 on A $\beta$ 42 production was proportionately greater leading to an increase in the ratio of A $\beta$ 42/total Ab. Indeed, the ratio of A $\beta$ 42/total A $\beta$  reaches a very high value of 42% in HEK293 cells cotransfected with Hu-Asp2 and APP-VF-KK.

### Example 9

#### Bacterial expression of human Asp2(a)

##### 5 *Expression of recombinant Hu-Asp2(a) in E. coli.*

Hu-Asp2(a) can be expressed in *E. coli* after addition of N-terminal sequences such as a T7 tag (SEQ ID No. 21 and No. 22) or a T7 tag followed by a caspase 8 leader sequence (SEQ ID No. 23 and No. 24). Alternatively, reduction of the GC content of the 5' sequence by site directed mutagenesis can be used to increase the  
10 yield of Hu-Asp2 (SEQ ID No. 25 and No. 26). In addition, Asp2(a) can be engineered with a proteolytic cleavage site (SEQ ID No. 27 and No. 28). To produce a soluble protein after expression and refolding, deletion of the transmembrane domain and cytoplasmic tail, or deletion of the membrane proximal region, transmembrane domain, and cytoplasmic tail is preferred. Any materials (vectors,  
15 host cells, etc.) and methods described herein to express Hu-Asp2(a) should in principle be equally effective for expression of Hu-Asp2(b).

##### *Methods*

PCR with primers containing appropriate linker sequences was used to assemble fusions of Asp2(a) coding sequence with N-terminal sequence modifications  
20 including a T7 tag (SEQ ID Nos. 21 and 22) or a T7-caspase 8 leader (SEQ ID Nos. 23 and 24). These constructs were cloned into the expression vector pet23a(+) [Novagen] in which a T7 promoter directs expression of a T7 tag preceding a sequence of multiple cloning sites. To clone Hu-Asp2 sequences behind the T7 leader of pet23a+, the following oligonucleotides were used for amplification of the selected  
25 Hu-Asp2(a) sequence: #553=GTGGATCCACCCAGCACGGCATCCGGCTG (SEQ ID No. 35), #554=GAAAGCTTTTCATGACTCATCTGTCTGTGGAATGTTG (SEQ ID No. 36) which placed BamHI and HindIII sites flanking the 5' and 3' ends of the insert, respectively. The Asp2(a) sequence was amplified from the full length Asp2(a) cDNA cloned into pcDNA3.1 using the Advantage-GC cDNA PCR [Clontech]  
30 following the manufacturer's supplied protocol using annealing & extension at 68°C in

the phosphorylated linkers together in 0.1 M NaCl-10 mM Tris, pH 7.4 they were ligated into unique Cla I and Sma I sites in Hu-Asp2 in the vector pTAC. For inducible expression using induction with isopropyl b-D-thiogalactopyranoside (IPTG), bacterial cultures were grown in LB broth in the presence of ampicillin at 100  
 5 ug/ml, and induced in log phase growth at an OD600 of 0.6-1.0 with 1 mM IPTG for 4 hour at 37°C. The cell pellet was harvested by centrifugation.

To create a vector in which the leader sequences can be removed by limited proteolysis with caspase 8 such that this liberates a Hu-Asp2 polypeptide beginning with the N-terminal sequence GSFV (SEQ ID Nos. 27 and 28), the following  
 10 procedure was followed. Two phosphorylated oligonucleotides containing the caspase 8 cleavage site IETD, #571=5' GATCGATGACTATCTCTGACTCTCCGCTGGACTCTGGTATCGAAACCGACG (SEQ ID No. 41) and #572= GATCCGTCGGTTTCGATACCAGAGTCCAGCGGAGAGTCAGAGATAGTCAT  
 15 C (SEQ ID No. 42) were annealed and ligated into pET23a+ that had been opened with BamHI. After transformation into JM109, the purified vector DNA was recovered and orientation of the insert was confirmed by DNA sequence analysis.

The following oligonucleotides were used for amplification of the selected Hu-Asp2(a) sequence:  
 20 #573=5'AAGGATCCTTTGTGGAGATGGTGGACAACCTG, (SEQ ID No. 43)  
 #554=GAAAGCTTTCATGACTCATCTGTCTGTGGAATGTTG (SEQ ID No. 44)  
 which placed BamHI and HindIII sites flanking the 5' and 3' ends of the insert, respectively. The Hu-Asp2(a) sequence was amplified from the full length Hu-Asp2(a) cDNA cloned into pcDNA3.1 using the Advantage-GC cDNA PCR  
 25 [Clontech] following the manufacturer's supplied protocol using annealing & extension at 68°C in a two-step PCR cycle for 25 cycles. The insert and vector were cut with BamHI and HindIII, purified by electrophoresis through an agarose gel, then ligated using the Rapid DNA Ligation kit [Boehringer Mannheim]. The ligation reaction was used to transform the *E. coli* strain JM109 [Promega] and colonies were  
 30 picked for the purification of plasmid (Qiagen, Qiaprep minispin) and DNA sequence

homogenized for 5 minutes at 3,000 rpm, volume adjusted to 250ml with cold water, then spun for 30 minutes. Weight of the resultant pellet was 17.75g.

Summary: Lysis of bacterial pellet in KCl solution, followed by centrifugation in a GSA rotor was used to initially prepare the pellet. The same solution was then  
5 used an additional three times for resuspension/homogenization. A final water wash/homogenization was then performed to remove excess KCl and EDTA.

*Solubilization of Recombinant Hu-Asp2(a):*

A ratio of 9-10ml/gram of pellet was utilized for solubilizing the rHuAsp2L from the  
10 pellet previously described. 17.75g of pellet was thawed, and 150ml of 8M guanidine HCl, 5mM  $\beta$ ME, 0.1% DEA, was added. 3M Tris was used to titrate the pH to 8.6. The pellet was initially resuspended into the guanidine solution using a 20 mm tissue homogenizer probe at 1000 rpm. The mixture was then stirred at 4°C for 1 hour prior to centrifugation at 12,500 rpm for 1 hour in GSA rotor. The resultant supernatant  
15 was then centrifuged for 30 minutes at 40,000 x g in an SS-34 rotor. The final supernatant was then stored at -20°C, except for 50 ml.

*Immobilized Nickel Affinity Chromatography of Solubilized Recombinant Hu-Asp2(a):*

20 The following solutions were utilized:

A) 6M Guanidine HCl, 0.1M NaP, pH 8.0, 0.01M Tris, 5mM  $\beta$ ME, 0.5mM Imidazole

A') 6M Urea, 20mM NaP, pH 6.80, 50mM NaCl

25 B') 6M Urea, 20mM NaP, pH 6.20, 50mM NaCl, 12mM Imidazole

C') 6M Urea, 20mM NaP, pH 6.80, 50mM NaCl, 300mM Imidazole

Note: Buffers A' and C' were mixed at the appropriate ratios to give intermediate concentrations of Imidazole.

The 50ml of solubilized material was combined with 50ml of buffer A prior to adding  
30 to 100-125ml Qiagen Ni-NTA SuperFlow (pre-equilibrated with buffer A) in a 5 x



(while stirring) with 200ml of (4°C) cold 20mM NaP, pH 6.8, 150mM NaCl. This dilution gave a final Urea concentration of 1M. This solution remained clear, even if allowed to set open to the air at room temperature (RT) or at 4°C .

5 After setting open to the air for 4-5 hours at 4°C, this solution was then dialyzed overnight against 20 mM NaP, pH 7.4, 150 mM NaCl, 20% glycerol. This method effectively removes the urea in the solution without precipitation of the protein.

Experiment 2:

10 Some of the 150-2 eluate was concentrated 2x on an Amicon Centriprep, 10,000 MWCO, then treated as in Experiment 1. This material also stayed in solution, with no visible precipitation.

Experiment 3:

15 89ml of the 150-2 eluate was spiked with 1M DTT, 3M Tris, pH 7.4 and DEA to a final concentration of 6mM, 50mM, and 0.1% respectively. This was diluted suddenly (while stirring) with 445 ml of (4°C) cold 20 mM NaP, pH 6.8, 150 mM NaCl. This solution appeared clear, with no apparent precipitation. The solution was removed to RT and stirred for 10 minutes prior to adding MEA to a final concentration of 0.1 mM. This was stirred slowly at RT for 1 hour. Cystamine and  
20 CuSO<sub>4</sub> were then added to final concentrations of 1 mM and 10 µM respectively. The solution was stirred slowly at RT for 10 minutes prior to being moved to the 4°C cold room and shaken slowly overnight, open to the air.

The following day, the solution (still clear, with no apparent precipitation) was centrifuged at 100,000 x g for 1 hour. Supernatants from multiple runs were pooled,  
25 and the bulk of the stabilized protein was dialyzed against 20mM NaP, pH 7.4, 150 mM NaCl, 20% glycerol. After dialysis, the material was stored at -20°C.

Some (about 10 ml) of the protein solution (still in 1M Urea) was saved back for biochemical analyses, and frozen at -20°C for storage.

Transient and stable expression of Hu-Asp2(a) $\Delta$ TM and Hu-Asp2(a) $\Delta$ TM(His)<sub>6</sub> in High 5 insect cells was performed using the insect expression vector pIZ/V5-His. The DNA inserts from the expression plasmids vectors pVL1393/Asp2(a), pVL1393/Asp2(a) $\Delta$ TM and pVL1393/Asp2(a) $\Delta$ TM(His)<sub>6</sub> were excised by double digestion with *Bam*HI and *Not*I and subcloned into *Bam*HI and *Not*I digested pIZ/V5-His using standard methods. The resulting expression plasmids, referred to as pIZ/Hu-Asp2 $\Delta$ TM and pIZ/Hu-Asp2 $\Delta$ TM(His)<sub>6</sub>, were prepared as described above.

For transfection, High 5 insect cells were cultured in High Five serum free medium supplemented with 10  $\mu$ g/ml gentamycin at 27 °C in sealed flasks. Transfections were performed using High five cells, High five serum free media supplemented with 10  $\mu$ g/ml gentamycin, and InsectinPlus liposomes (Invitrogen, Carlsbad, CA) using standard methods.

For large scale transient transfections,  $1.2 \times 10^7$  high five cells were plated in a 150 mm tissue culture dish and allowed to attach at room temperature for 15-30 minutes. During the attachment time the DNA/ liposome mixture was prepared by mixing 6 ml of serum free media, 60  $\mu$ g Hu-Asp2(a) $\Delta$ TM/pIZ (+/- His) DNA and 120  $\mu$ l of Insectin Plus and incubating at room temperature for 15 minutes. The plating media was removed from the dish of cells and replaced with the DNA/liposome mixture for 4 hours at room temperature with constant rocking at 2 rpm. An additional 6 ml of media was added to the dish prior to incubation for 4 days at 27 °C in a humid incubator. Four days post transfection the media was harvested, clarified by centrifugation at 500 x g, assayed for Hu-Asp2(a) expression by Western blotting. For stable expression, the cells were treated with 50  $\mu$ g/ml Zeocin and the surviving pool used to prepared clonal cells by limiting dilution followed by analysis of the expression level as noted above.

#### *Purification of Hu-Asp2(a) $\Delta$ TM and Hu-Asp2(u) $\Delta$ TM(His)<sub>6</sub>*

Removal of the transmembrane segment from Hu-Asp2(a) resulted in the secretion of the polypeptide into the culture medium. Following protein production

cationic liposome DOTAP as recommended by the supplier (Roche, Indianapolis, IN). The cells were treated with the plasmid DNA/liposome mixtures for 15 hours and then the medium replaced with growth medium containing 500 Units/ml hygromycin B. In the case of pcDNA3.1(+)/Hygro/Hu-Asp2(a) or (b) transfected CHO-K1 cells, individual hygromycin B-resistant cells were cloned by limiting dilution. Following clonal expansion of the individual cell lines, expression of Hu-Asp2(a) or Hu-Asp2(b) protein was assessed by Western blot analysis using a polyclonal rabbit antiserum raised against recombinant Hu-Asp2 prepared by expression in *E. coli*. Near confluent dishes of each cell line were harvested by scraping into PBS and the cells recovered by centrifugation. The cell pellets were resuspended in cold lysis buffer (25 mM Tris-HCl (pH 8.0)/5 mM EDTA) containing protease inhibitors and the cells lysed by sonication. The soluble and membrane fractions were separated by centrifugation (105,000 x g, 60 min) and normalized amounts of protein from each fraction were then separated by SDS-PAGE. Following electrotransfer of the separated polypeptides to PVDF membranes, Hu-Asp-2(a) or Hu-Asp2(b) protein was detected using rabbit anti-Hu-Asp2 antiserum (1/1000 dilution) and the antibody-antigen complexes were visualized using alkaline phosphatase conjugated goat anti-rabbit antibodies (1/2500). A specific immunoreactive protein with an apparent Mr value of 65 kDa was detected in pcDNA3.1(+)/Hygro/Hu-Asp2 transfected cells and not mock-transfected cells. Also, the Hu-Asp2 polypeptide was only detected in the membrane fraction, consistent with the presence of a signal peptide and single transmembrane domain in the predicted sequence. Based on this analysis, clone #5 had the highest expression level of Hu-Asp2(a) protein and this production cell lines was scaled up to provide material for purification.

25 *Purification of recombinant Hu-Asp-2(a) from CHO-K1/Hu-Asp2 clone #5*

In a typical purification, clone #5 cell pellets derived from 20 150 mm dishes of confluent cells, were used as the starting material. The cell pellets were resuspended in 50 ml cold lysis buffer as described above. The cells were lysed by polytron homogenization (2 x 20 sec) and the lysate centrifuged at 338,000 x g for 20 minutes. The membrane pellet was then resuspended in 20 ml of cold lysis buffer

Asp2(a) (1-5  $\mu$ g protein) for various times at 37°C. The reaction products were quantified by RP-HPLC using a linear gradient from 0-70 B over 30 minutes (A=0.1% TFA in water, B=0.1%TFA/10%water/90%AcCN). The elution profile was monitored by absorbance at 214 nm. In preliminary experiments, the two product peaks which eluted before the intact peptide substrate, were confirmed to have the sequence DAEFR and SEVNL using both Edman sequencing and MADLI-TOF mass spectrometry. Percent hydrolysis of the peptide substrate was calculated by comparing the integrated peak areas for the two product peptides and the starting material derived from the absorbance at 214 nm. The sequence of cleavage/hydrolysis products was confirmed using Edman sequencing and MADLI-TOF mass spectrometry.

The behavior of purified Asp2(a) in the proteolysis assays was consistent with the prior anti-sense studies which indicated that Asp2(a) possesses  $\beta$ -secretase activity. Maximal proteolysis was seen with the Swedish  $\beta$ -secretase peptide, which, after 6 hours, was about 10-fold higher than wild type APP.

The specificity of the protease cleavage reaction was determined by performing the  $\beta$ -secretase assay in the presence of 8  $\mu$ M pepstatin A and the presence of a cocktail of protease inhibitors (10  $\mu$ M leupeptin, 10  $\mu$ M E64, and 5 mM EDTA). Proteolytic activity was insensitive to both the pepstatin and the cocktail, which are inhibitors of cathepsin D (and other aspartyl proteases), serine proteases, cysteinyl proteases, and metalloproteases, respectively.

Hu-Asp2(b) when similarly expressed in CHO cells and purified using identical conditions for extraction with  $\beta$ -octylglucoside and sequential chromatography over Mono Q and Mono S also cleaves the Swedish  $\beta$ -secretase peptide in proteolysis assays using identical assay conditions.

Collectively, this data establishes that both forms of Asp2 (Hu-Asp2(a) and Hu-Asp2(b)) act directly in cell-free assays to cleave synthetic APP peptides at the  $\beta$ -secretase site, and that the rate of cleavage is greatly increased by the Swedish KM-NL mutation that is associated with Alzheimer's disease.

Table 5.

**Asp1 stimulates basal release of sAPP $\alpha$  from HEK293 cells after cotransfection with APP-KK.**

5	Transfection	sAPP $\alpha$ $\mu\text{g/ml}$	Fold Increase	A $\beta$ 40 $\text{pg/ml}$	Fold Decrease
	Asp1	3.5 + 1.1	+3.5	113 + 7	-2.8
	pcDNA	1.0 + 0.2		321 + 18	

Specific methods used were as follows. The full length Asp1 cDNA was  
 10 cloned into the vector pcDNA3.1/hygro+(Invitrogen) for transfection studies as  
 previously described (Yan *et al.*, (1999) *Nature* 402: 533-537). The APP-KK cDNA  
 was cloned into the vector pIRES (Clontech) also as previously described. HEK293  
 cells were transfected with expression constructs using the Lipofectamine Plus reagent  
 from Gibco/BRL. Cells were seeded in 24 well tissue culture plates to a density of  
 15 70-80% confluence. Four wells per plate were transfected with 2  $\mu\text{g}$  DNA (3:1,  
 APP:Asp1 or empty pcDNA3.1/hygro+ vector), 8  $\mu\text{l}$  Plus reagent, and 4  $\mu\text{l}$   
 Lipofectamine in OptiMEM. OptiMEM was added to a total volume of 1 ml,  
 distributed 200  $\mu\text{l}$  per well and incubated 3 hours. Care was taken to hold constant the  
 ratios of the two plasmids used for cotransfection as well as the total amount of DNA  
 20 used in the transfection. The transfection media was replaced with DMEM  
 supplemented with 10% FBS and NaPyruvate, with antibiotic/antimycotic and the  
 cells were incubated under normal conditions (37°, 5% CO<sub>2</sub>) for 48 hours. The  
 conditioned media were removed to polypropylene tubes and stored at -80°C until  
 assayed for the content of sAPP $\alpha$  or A $\beta$ 40/A $\beta$ 42 by enzyme-linked immunosorbent  
 25 assay (EIA) as described above in Example 6. The A $\beta$  EIA followed the protocol of  
 Pirttila *et al.* (*Neuro. Lett.* (1999) 249: 21-4) using the 6E10 monoclonal antibody  
 (Senetek) as a capture antibody and biotinylated rabbit antiserum 162 or 165 (New  
 York State Institute for Basic Research, Staten Island, NY) for detection of A $\beta$ 40 and  
 A $\beta$ 42, respectively. The 6E10 antibody recognizes residues 1-16 of the A $\beta$  peptide.  
 30 The sAPP $\alpha$  EIA used LN27 antibody as a capture antibody and biotinylated 6E10 for

The reaction product was digested to completion with HindIII-XhoI and ligated into the expression vector pIB (Invitrogen) to yield the pIB/Asp-1ΔTM(His)<sub>6</sub> construct. Creation of recombinant baculovirus and infection of sf9 insect cells was performed using standard methods known in the art. Sf9 cells were transfected with either the pIB vector alone or the pIB/Asp-1ΔTM(His)<sub>6</sub> construct utilizing Insectin Plus reagent (Invitrogen) according to the manufacturer's instructions. After the transfection, the cells were cultured in High Five serum-free media (Invitrogen) for 3 days. Subsequently, the conditioned medium was harvested and subjected to Western blot analysis. This analysis revealed specific expression and secretion of immunoreactive Asp-1ΔTM(His)<sub>6</sub> polypeptide into the extracellular medium. The secreted proteins were detected on the Western blot with either the India probe (Pierce Chemicals) specific for the hexahistidine sequence tag or using a rabbit polyclonal antiserum. The polyclonal antisera (denoted as UP-199) was generated by injecting rabbits with recombinant Asp-1ΔTM(His)<sub>6</sub> (SEQ ID NO: 66). This recombinant peptide was prepared by heterologous expression in *E. coli*. The UP-199 antibody recognizes the processed form of Asp-1ΔTM.

Direct analysis with the polyclonal antiserum (UP-199) revealed an immunoreactive band of the expected molecular weight (50 kDa) only in pIB/Asp-1ΔTM(His)<sub>6</sub> transfected cells. This signal was significantly enhanced in concentrated conditioned medium. A similar pattern was obtained using the India probe. No signal was detected in conditioned medium derived from mock-transfected cells using either UP-199 antisera or the India probe.

Based on this result, transient and stable transfections of the pIB/Asp-1ΔTM(His)<sub>6</sub> construct in sf9 insect cells were carried out as described above. Four days post transient transfection, the culture medium was collected to provide material for further characterization. In parallel, sf9 cells were stably transfected with the pIB/Asp-1ΔTM(His)<sub>6</sub> construct and cultured in High Five serum-free medium (Invitrogen) supplemented with 50 μg/ml blasticidin for approximately 2 weeks. After blasticidin selection, the resistant pool of cells was expanded to provide a stable source of conditioned medium for Asp-1ΔTM(His)<sub>6</sub> purification.

Biotech) gradient elution containing increasing concentrations (0 -0.5 M) of NaCl (Buffer A: 25 mM Tris-HCl (pH 8.0) and Buffer B: 25 mM Tris-HCl (pH 8.0)/ 0.5 M NaCl). The elution profile was determined by Western blot analysis which indicated immunoreactive fractions as those displaying immunoreactivity with the UP-199 antisera. NuPAGE gel analysis with silver staining demonstrated that the material prepared in this manner was >90% pure. The immunoactive fractions eluted off the MonoQ anion exchange column were pooled, dialyzed with 25 mM HEPES-Na<sup>+</sup> (pH 8.0), and stored at 4°C until further analysis.

10 *Acid-activation of recombinant Asp-1 TM(His)<sub>6</sub>*

Recombinant Asp-1ΔTM(His)<sub>6</sub> migrated with an apparent molecular weight of 50 kD. Direct N-terminal sequence analysis carried out by automated Edman degradation for 20 cycles revealed a unique sequence beginning at Glu<sup>1</sup> (SEQ ID NO: 67), confirming the identity of the recombinant protein. Computer assisted prediction of the signal peptidase cleavage site indicated that the pro-form should initiate at Ala<sup>1</sup>, suggesting either an unusual processing site by the signal peptidase during secretion or an additional processing step that removes an additional two amino acid residues.

To investigate the mechanism of pro-Asp-1ΔTM(His)<sub>6</sub> activation, aliquots of the purified protein were incubated in various acidic environments with pH values ranging from 3.0-8.0 at 37°C for 2 hours. Subsequently, the recombinant proteins were analyzed by Western blot. A faster migrating polypeptide species was detected after incubation at pH values of 4.0, 4.5 and 5.0. The polypeptide migration was unaltered after incubation in environments which were either more acidic (pH 3.0 and 3.5) or more basic (pH 6.0, 7.0, and 8.0). Sequence analysis of this faster migrating species revealed that it initiated exclusively at Ala<sup>43</sup>, consistent with removal of a 42 amino acid residue segment of the pro-peptide that was induced by treatment of the pro-enzyme at pH 4.5. The predicted amino acid sequence of the acid processed form of Asp-1ΔTM(His)<sub>6</sub> is set out as SEQ ID NO: 68.

To purify the acid-activated form of Asp-1ΔTM(His)<sub>6</sub>, the Asp-1ΔTM(His)<sub>6</sub> post-IMAC pool (generated as described above) was dialyzed to pH 4.5 and then

of a peptide sequences (surrounding Ala<sup>43</sup>) that could be useful for performing screening assays to identify modulators of Asp1 activity. This idea is explored in greater detail in Example 15.

### Example 15

#### Development of an enzymatic assay for Asp-1ΔTM(His)<sub>6</sub>

The relationship between Asp1 and APP processing was explored by determining if APP α-secretase, APP β-secretase, or APP γ-secretase peptide substrates were cleaved by recombinant Asp-1ΔTM(His)<sub>6</sub>. These peptide substrates included the α-secretase specific substrates Aβ<sub>10-20</sub> and Aβ<sub>12-28</sub>, the β-secretase specific substrates PHA-95812E (SEVKMDAEFR; SEQ ID NO: 64) and PHA-247574E (SEVNLDAEFR; SEQ ID NO: 63), and γ-secretase specific substrate PHA-179111E (RRGGVVIATVIVGER; SEQ ID NO: 76). Each reaction consisted of incubating a peptide substrate (100 nM) with recombinant Asp-1ΔTM(His)<sub>6</sub> for 15 hours at pH 4.5 at 37°C. Reaction products were quantified by RP-HPLC at A<sup>214nm</sup>. The elution profiles for Asp-1ΔTM(His)<sub>6</sub> were compared to those obtained from parallel Asp1 experiments. The identity of the cleavage products was determined by MADLI-TOF mass spectrometry. Table 6 summarizes the Asp1 substrates and indicates the cleavage site.

Table 6

#### Substrate Preferences of Asp-1ΔTM

<u>P4</u>	<u>P3</u>	<u>P2</u>	<u>P1</u>		<u>P1'</u>	<u>P2'</u>	<u>P3'</u>	<u>P4'</u>		SEQ ID NO:
G	L	A	L	1	A	L	E	P	Self Activation	69
E	V	K	M	1	D	A	E	F	β-Secretase, WT	70
E	V	N	L	1	D	A	E	F	β-Secretase, Sw	71
L	V	F	F	1	A	E	D	V	Aβ <sub>12-28</sub> (α-Secretase)	72
K	L	V	F	1	F	A	E	D	Aβ <sub>12-28</sub> (α-Secretase)	73



processing sites. These substrates are useful in screening assays for identification of modulators of Asp1 proteolytic activity.

In particular, production of A $\beta$  species through the processing of APP at  $\beta$ - and  $\gamma$ -secretase sites may play a central role in Alzheimer's disease pathogenesis, and processing at the  $\alpha$ -secretase site may have a protective role and may prevent A $\beta$  production. Thus, a therapeutic and/or prophylactic indication exists for molecules that can increase Asp1  $\alpha$ -secretase activity and/or decrease Asp1  $\beta$ -secretase activity *in vivo*. The present invention includes screening assays for such modulators, and the foregoing substrate peptides are useful in such assays.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the invention. The entire disclosure of all publications cited herein are hereby incorporated by reference.

7. A method according to any one of claims 1-6, wherein the hu-Asp1 amino acid sequence lacks amino terminal amino acids 1-62 of SEQ ID NO: 2.

8. A method according to claims 1 or 2, wherein the contacting step comprises growing a cell transfected or transformed with a polynucleotide encoding hu-Asp1 protein or a fragment thereof that retains hu-Asp1  $\alpha$ -secretase activity, wherein the cell is grown under conditions in which the cell expresses the hu-Asp1 protein in the presence of the APP substrate.

9. A method of claim 8, wherein said cell expresses a polynucleotide encoding an APP substrate containing an  $\alpha$ -secretase cleavage site, and wherein the contacting step comprises growing the cell under conditions in which the cell expresses the hu-Asp1 protein and the APP substrate.

10. A method of any one of claims 1-9, wherein the APP substrate  $\alpha$ -secretase cleavage site comprises the amino acid sequence LVFFAEDF or KLVFFAED.

11. A method of any one of claims 1-10, wherein the APP substrate comprises a human APP isoform and further comprises a carboxy-terminal di-lysine.

12. A method of claims 10 or 11, wherein the APP substrate comprises a detectable label.

13. A method of claim 12, wherein the detectable label is selected from the group consisting of radioactive labels, chemiluminescent labels, enzymatic labels, chemiluminescent labels and fluorescent labels.

21. A method for assaying hu-Asp1 proteolytic activity comprising the steps of:
- (a) contacting hu-Asp1 protein with an hu-Asp1 substrate according to claim 18, 19, or 20 under acidic conditions, and
  - 5 (b) determining the level of hu-Asp1 proteolytic activity.
22. A method according to claim 20, wherein the hu-Asp1 protein comprises a polypeptide produced in cell transformed or transfected with a polynucleotide comprising the nucleotide sequence that encodes hu-Asp1.
- 10 23. A method of claim 21, wherein the hu-Asp1 protein is purified and isolated from said cell.
24. A method according to claims 22 and 23, wherein the nucleotide
- 15 sequence encodes a polypeptide that comprises the hu-Asp1 amino acid sequence set forth in SEQ ID NO: 2 or a fragment thereof, wherein said fragment retains proteolytic activity.
25. A purified polynucleotide comprising a nucleotide sequence encoding
- 20 a polypeptide that comprises a fragment of a hu-Asp-1 protein, wherein said nucleotide sequence lacks the sequence that encodes the transmembrane domain of said hu-Asp1 protein, and wherein the hu-Asp1 polypeptide fragment encoded by said polynucleotide has hu-Asp1  $\alpha$ -secretase activity.
- 25 26. A polynucleotide of claim 25, wherein the polypeptide comprises a fragment of the hu-Asp1 amino acid sequence set forth in SEQ ID NO: 2, and wherein the polypeptide lacks the transmembrane domain amino acids 469-492 of SEQ ID NO: 2.

35. A purified polypeptide comprising a fragment of a hu-Asp1 protein, wherein said polypeptide lacks the hu-Asp1 transmembrane domain of said hu-Asp1 protein and retains hu-Asp1  $\alpha$ -secretase activity.

5           36. A polypeptide of claim 35, wherein said polypeptide comprises a fragment of hu-Asp1 having the amino acid sequence set forth in SEQ ID NO: 2 and wherein said polypeptide lacks the transmembrane domain amino acids 469-492 of SEQ ID NO: 2.

10           37. A polypeptide according to claim 35 or 36, wherein said polypeptide further lacks cytoplasmic domain amino acids 493-518 of SEQ ID NO: 2.

            38. A polypeptide according to any one of claims 35-37, which further lacks amino terminal amino acids 1-62 of SEQ ID NO: 2.

15           39. A polypeptide comprising a fragment of hu-Asp1 having the amino acid sequence set forth in SEQ ID NO: 2 and wherein said polypeptide lacks the amino terminal amino acids 1-62 of SEQ ID NO: 2 and retains APP processing activity.

20           40. A polypeptide comprising an amino acid sequence at least 95% identical to a fragment of hu-Asp1 protein, wherein said polypeptide and said fragment lack a transmembrane domain and retain hu-Asp1  $\alpha$ -secretase activity.

25           41. A polypeptide comprising an amino acid sequence at least 95% identical to a fragment of hu-Asp1 protein, wherein said polypeptide and said fragment lack the amino terminal amino acids corresponding to the pro-peptide domain of hu-Asp1 and retain APP processing activity.

30

TCCACAAATGCGCTGGTGATCGGTGCCACGGTGATGGAGGGCTTCTACGTCATCTTCGAC  
S T N A L V I G A T V M E G F Y V I F D

AGAGCCCAGAAGAGGGTGGGCTTCGCAGCGAGCCCCTGTGCAGAAATTGCAGGTGCTGCA

FIGURE 2 (1)

ATGGCCCAAGCCCTGCCCTGGCTCCTGCTGTGGATGGGCGCGGGAGTGCTGCCCTGCCAC  
M A Q A L P W L L L W M G A G V L P A H  
GGCACCAGCACGGCATCCGGCTGCCCTGCGCAGCGGCTGGGGGGCGCCCCCTGGGG  
G T Q H G I R L P L R S G L G G A P L G  
CTGCGGCTGCCCCGGGAGACCGACGAAGAGCCCGAGGAGCCCGCGCGAGGGGCAGCTTT  
L R L P R E T D E E P E E P G R R G S F  
GTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGGCAGGGCTACTACGTGGAGATGACC  
V E M V D N L R G K S G Q G Y Y V E M T  
GTGGGCAGCCCCCGCAGACGCTCAACATCCTGGTGGATACAGGCAGCAGTAACCTTTGCA  
V G S P P Q T L N I L V D T G S S N F A  
GTGGGTGCTGCCCCCACCCCTTCCTGCATCGCTACTACCAGAGGCAGCTGTCCAGCACA  
V G A A P H P F L H R Y Y Q R Q L S S T  
TACCGGGACCTCCGGAAGGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGAAGGGGAG  
Y R D L R K G V Y V P Y T Q G K W E G E  
CTGGGCACCGACCTGGTAAGCATCCCCATGGCCCCAACGTCACTGTGCGTGCCAACATT  
L G T D L V S I P H G P N V T V R A N I  
GCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCCAACTGGGAAGGCATCCTG  
A A I T E S D K F F I N G S N W E G I L  
GGGCTGGCCTATGCTGAGATTGCCAGGCTTTGTGGTGCTGGCTTCCCCCTCAACCAGTCT  
G L A Y A E I A R L C G A G F P L N Q S  
GAAGTGCTGGCCTCTGTCCGAGGGAGCATGATCATTGGAGGTATCGACCACTCGCTGTAC  
E V L A S V G G S M I I G G I D H S L Y  
ACAGGCAGTCTCTGGTATACACCCATCCGGCGGGAGTGGTATTATGAGGTGATCATTTGTG  
T G S L W Y T P I R R E W Y Y E V I I V  
CGGGTGGAGATCAATGGACAGGATCTGAAAATGGACTGCAAGGAGTACAACCTATGACAAG  
R V E I N G Q D L K M D C K E Y N Y D K  
AGCATTGTGGACAGTGGCACCACCAACCTTCGTTTGCCCAAGAAAGTGTTPGAAGCTGCA  
S I V D S G T T N L R L P K K V F E A A  
GTCAAATCCATCAAGGCAGCCTCCTCCACGGAGAAGTTCCCTGATGGTTTCTGGCTAGGA  
V K S I K A A S S T E K F P D G F W L G  
GAGCAGCTGGTGTGCTGGCAAGCAGGCACCACCCCTTGGAACATTTTCCAGTCATCTCA  
E Q L V C W Q A G T T P W N I F P V I S  
CTCTACCTAATGGGTGAGGTTACCAACCAGTCCTTCCGCATCACCATCCTTCCGCAGCAA  
L Y L M G E V T N Q S F R I T I L P Q Q  
TACCTGCGGCCAGTGGAAGATGTGGCCACGTCCCAAGACGACTGTTACAAGTTTGCCATC

FIGURE 3 (1)

ATGCCCAAGCCCTGCCCTGGCTCCTGCTGTGGATGGGCGCGGAGTGCTGCCCTGCCAC  
M A Q A L P W L L L W M G A G V L P A H  
GGCACCACAGCACGGCATCCGGCTGCCCTGCGCAGCGGCTGGGGGGCGCCCCCTGGGG  
G T Q H G I R L P L R S G L G G A P L G  
CTGCGGCTGCCCGGGAGACCGACGAAGAGCCCGAGGAGCCCGGCCGGAGGGGCAGCTTT  
L R L P R E T D E E P E E P G R R G S F  
GTGGAGATGGTGGACAACCTGAGGGGCAAGTCGGGGCAGGGCTACTACGTGGAGATGACC  
V E M V D N L R G K S G Q G Y Y V E M T  
GTGGGCAGCCCCCGCAGACGCTCAACATCCTGGTGGATACAGGCAGCAGTAACCTTTGCA  
V G S P P Q T L N I L V D T G S S N F A  
GTGGGTGCTGCCCCCACCCCTTCCTGCATCGCTACTACCAGAGGCAGCTGTCCAGCACA  
V G A A P H P F L H R Y Y Q R Q L S S T  
TACCGGGACCTCCGGAAGGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGGAAGGGGAG  
Y R D L R K G V Y V P Y T Q G K W E G E  
CTGGGCACCGACCTGGTAAGCATCCCCATGGCCCCAACGTCACTGTGCGTGCCAACATT  
L G T D L V S I P H G P N V T V R A N I  
GCTGCCATCACTGAATCAGACAAGTTCTTCATCAACGGCTCCAAGTGGGAAGGCATCCTG  
A A I T E S D K F F I N G S N W E G I L  
GGGCTGGCCTATGCTGAGATTGCCAGGCCTGACGACTCCCTGGAGCCTTTCTTTGACTCT  
G L A Y A E I A R P D D S L E P F F D S  
CTGGTAAAGCAGACCCACGTTCCCAACCTCTTCTCCCTGCAGCTTTGTGGTGTGGCTTC  
L V K Q T H V P N L F S L Q L C G A G F  
CCCCCAACCAGTCTGAAGTGCTGGCCTCTGTGGAGGGAGCATGATCATTGGAGGTATC  
P L N Q S E V L A S V G G S M I I G G I  
GACCACTCGCTGTACACAGGCAGTCTCTGGTATACACCCATCCGGCGGGAGTGGTATTAT  
D H S L Y T G S L W Y T P I R R E W Y Y  
GAGGTCATCATTGTGCGGGTGGAGATCAATGGACAGGATCTGAAAATGGACTGCAAGGAG  
E V I I V R V E I N G Q D L K M D C K E  
TACAACTATGACAAGAGCATTGTGGACAGTGGCACCACCAACCTTCGTTTGCCCAAGAAA  
Y N Y D K S I V D S G T T N L R L P K K  
GTGTTTGAAGCTGCAGTCAAATCCATCAAGGCAGCCTCCTCCACGGAGAAGTTCCCTGAT  
V F E A A V K S I K A A S S T E K F P D

FIGURE 4

ATGGCCCCAGCGCTGCACTGGCTCCTGCTATGGGTGGGCTCGGGAATGCTGCCTGCCAG  
 M A P A L H W L L L W V G S G M L P A Q  
 GGAACCCATCTCGGCATCCGGCTGCCCTTCGAGCGGCTGGCAGGGCCACCCCTGGGC  
 G T H L G I R L P L R S G L A G P P L G  
 CTGAGGCTGCCCCGGGAGACTGACGAGGAATCGAGGAGCTGGCCGGAGAGGCAGCTT  
 L R L P R E T D E E S E E P G R R G S F  
 GTGGAGATGGTGGACAACCTGAGGGGAAAGTCCGCCAGGGCTACTATGTGGAGATGACC  
 V E M V D N L R G K S G Q G Y Y V E M T  
 GTAGGCAGCCCCACAGACGCTCAACATCCTGGTGGACACGGGCAGTAGTAACCTTTGCA  
 V G S P P Q T L N I L V D T G S S N P A  
 GTGGGGCTGCCCCACACCTTTCTGTCATCGCTACTACCAGAGGCAGCTGTCCAGCACA  
 V G A A P H P F L H R Y Y Q R Q L S S T  
 TATCGAGACCTCCGAAAGGTGTGTATGTGCCCTACACCCAGGGCAAGTGGGAGGGGAA  
 Y R D L R K G V Y V P Y T Q G K W E G E  
 CTGGGCACCGACCTGGTGGAGCATCCCTCATGGCCCCAACGTCACGTGTGGGTGCCAACATT  
 L G T D L V S I P H G P N V T V R A N I  
 GCTGCCATCACTGAATCGGACAAGTTCTTCATCAATGGTTCCAACCTGGGAGGGCATCCTA  
 A A I T E S D K F F I N G S N W E G I L  
 GGGCTGGCCTATGCTGAGATTGCCAGGCCCGACGACTCTTTGGAGCCCTTCTTTGACTCC  
 G L A Y A E I A R P D D S L E P F F D S  
 CTGTTGAAGCAGACCCACATTCCCAACATCTTTCCCTGCAGCTCTGTGGCGCTGGCTTC  
 L V K Q T H I P N I F S L Q L C G A G F  
 CCCCTCAACCAGACCGAGGCAGTGGCTCGGTGGGAGGGAGCATGATCATTGGTGGTATC  
 P L N Q T E A L A S V G G S M I I G G I  
 GACCACTCGCTATACACGGGCAGTCTCTGGTACACCCATCCGGCGGGAGTGGTATTAT  
 D H S L Y T G S L W Y T P I R R E W Y Y  
 GAAGTGATCATGTACGTGTGGAAATCAATGGTCAAGATCTCAAGATGGACTGCAAGGAG  
 E V I I V R V E I N G Q D L K M D C K E  
 TACAACACGACAAGAGCATTTGTGGACAGTGGGACCACCAACCTTCGCTTGCCCAAGAAA  
 Y N Y D K S I V D S G T T N L R L P K K  
 GTATTTGAAGCTGCCGTCAAGTCCATCAAGGCAGCCTCCTCGACGGAGAAGTTCCGGAT  
 V F E A A V K S I K A A S S T E K P P D  
 GGCTTTTGGCTAGGGGAGCAGCTGGTGTGCTGGCAAGCAGGCACGACCCCTTGGAACATT  
 G F W L G E Q L V C W Q A G T T P W N I  
 TTCCAGTCATTTCATTACCTCATGGGTGAAGTCACCAATCAGTCTTCGCGATCACC  
 F P V I S L Y L M G E V T N Q S F R I T  
 ATCCTTCTCAGCAATACCTACGGCCGGTGGAGGACGTGGCCACGTCCCAAGACGACTGT  
 I L P Q Q Y L R P V E D V A T S Q D D C  
 TACAAGTTCTGCTGTCTCACAGTCATCCACGGGCAGTGTATGGGAGCCGTATCATGGAA  
 Y K F A V S Q S S T G T V M G A V I M E  
 GGTTTCTATGTCGTCTTCGATCGAGCCCGAAAGCGAATGGCTTTGCTGTGAGCGCTTGC  
 G F Y V V F D R A R K R I G F A V S A C  
 CATGTGCACGATGAGTTCAGGACGGCGGAGTGGAAAGTCCGTTTGTACGGCAGACATG  
 H V H D E F R T A A V E G P F V T A D M  
 GAAGACTGTGGCTACAACATTCCCCAGACAGATGAGTCAACACTTATGACCATAGCCTAT  
 E D C G Y N I P Q T D E S T L M T I A Y  
 GTCATGGCGGCCATCTGCGCCCTCTTCATGTTGCCACTCTGCCCTCATGGTATGTACGTGG  
 V M A A I C A L F M L P L C L M V C Q W  
 CGCTGCGCTGCGTTGCGCCACCGACGATGACTTTGCTGATGACATCTCCCTGCTC  
 R C L R C L R H Q H D D F A D D I S L L  
 AAGTAAGGAGGCTCGTGGGCAGATGATGGAGACGCCCTGGACCACATCTGGGTGGTTCC  
 K  
 CTTTGGTCACATGAGTTGGAGCTATGGATGGTACCTGTGGCCAGAGCACCTCAGGACCCCT  
 CACCAACCTGCCAATGCTTCTGGCGTGACAGAACAGAGAAATCAGGCAAGCTGGATTACA  
 GGGCTTGCACCTGTAGGACACAGGAGAGGGAAGGAAGCAGCGTTCTGGTGGCAGGAATAT  
 CCTTAGGCACCACAACTTGAGTTGGAAATTTTGTGCTTGAAGCTTCAGCCCTGACCCCT  
 CTGCCCAGCATCCTTAGAGTCTCAACCTAAAGTATTCTTTATGTCCTTCAGAAGTAC



**FIGURE 5**

1	MAQALPWL	LLWMGAGV	LPAHGTO	HGIRLPL	RSGLAGP	PLGLRLP	PRETDEE	50
1	MAPALHW	LLLVGSG	MLPAQGT	HLGIRL	PLRSGL	AGPPLGL	RLLPRET	50
51	PEEPGR	RRGSFV	EMVDNL	RGKSGQ	GYVEMT	VGSPPT	LNILVD	100
51	SEEPGR	RRGSFV	EMVDNL	RGKSGQ	GYVEMT	VGSPPT	LNILVD	100
101	VGAAPH	PFLHRY	YQRQLS	STYRDL	RKGVVP	PTYQGK	WEGELG	150
101	VGAAPH	PFLHRY	YQRQLS	STYRDL	RKGVVP	PTYQGK	WEGELG	150
151	GNVTVR	ANIAAI	TESDKF	FINGSN	WEGILG	LAYABIA	RPDDSL	200
151	GNVTVR	ANIAAI	TESDKF	FINGSN	WEGILG	LAYABIA	RPDDSL	200
201	LVKQTH	VPNLF	SLQLCG	AGFPLN	QSEVLAS	VGGSMI	IGGIDH	250
201	LVKQTH	IPNIF	SLQLCG	AGFPLN	QTEALAS	VGGSMI	IGGIDH	250
251	YTPIRR	EWWYEV	IIVRVE	INGQDL	KMDCKE	YNYDKS	IVDSGT	300
251	YTPIRR	EWWYEV	IIVRVE	INGQDL	KMDCKE	YNYDKS	IVDSGT	300
301	VFEAAV	KSIIKA	ASSTEK	FPDGF	WLGEQL	VCWQAG	TPWNIF	350
301	VFEAAV	KSIIKA	ASSTEK	FPDGF	WLGEQL	VCWQAG	TPWNIF	350
351	EVTNQSF	RITILP	QQYLRP	VEDVAT	SQDDCY	KFAISQ	SSSTGT	400
351	EVTNQSF	RITILP	QQYLRP	VEDVAT	SQDDCY	KFAVSQ	SSSTGT	400
401	GFYVVF	DRARKR	IGFAVS	ACHVHD	EFRTAA	VEGPPV	TLDMED	450
401	GFYVVF	DRARKR	IGFAVS	ACHVHD	EFRTAA	VEGPPV	TADMED	450
451	DESTLMT	IIAYVM	AAICAL	FMLPLC	LMVCQW	RCLRCL	RQHDDF	500
451	DESTLMT	IIAYVM	AAICAL	FMLPLC	LMVCQW	RCLRCL	RHQHDD	500
501	K	501						
501	K	501						

FIGURE 6 (2)

R P V E D V A T S Q D D C Y K F A I S Q  
TCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAGGGCTTCTACGTTGTCTTTGAT  
S S T G T V M G A V I M E G F Y V V F D  
CGGGCCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGCCATGTGCACGATGAGTTCAGG  
R A R K R I G F A V S A C H V H D E F R  
ACGGCAGCGGTGGAAGGCCCTTTGTACCTTGGACATGGAAGACTGTGGCTACAACATT  
T A A V E G P F V T L D M E D C G Y N I  
CCACAGACAGATGAGTCATGA  
P Q T D E S \*

FIGURE 7 (2)

CAGTCCTCCGCATCACCATCCTTCGGCAGCAATACCTGCGGCCAGTGGGAAGATGTGGCC  
Q S F R I T I L P Q Q Y L R P V E D V A

ACGTCCCAAGACGACTGTTACAAGTTTGCCATCTCACAGTCATCCACGGGCACTGTTATG  
T S Q D D C Y K F A I S Q S S T G T V M

GGAGCTGTTATCATGGAGGGCTTCTACGTTGTCTTTGATCGGGCCCGAAAACGAATTGGC  
G A V I M E G F Y V V F D R A R K R I G

TTTGCTGTCAGCGCTTGCCATGTGCACGATGAGTTCAGGACGGCAGCGGTGGAAGGCCCT  
F A V S A C H V H D E F R T A A V E G P

TTTGTACCTTGGACATGGAAGACTGTGGCTACAACATTCCACAGACAGATGAGTCATGA  
F V T L D M E D C G Y N I P Q T D E S \*

FIGURE 8 (2)

TACAAGTTTGCCATCTCACAGTCATCCACGGGCACTGTTATGGGAGCTGTTATCATGGAG  
Y K F A I S Q S S T G T V M G A V I M E -  
GGCTTCTACGTTGTCTTTGATCGGGCCGAAAACGAATTGGCTTTGCTGTCAGCGCTTGC  
G F Y V V F D R A R K R I G F A V S A C -  
CATTAG  
H \*

FIGURE 10

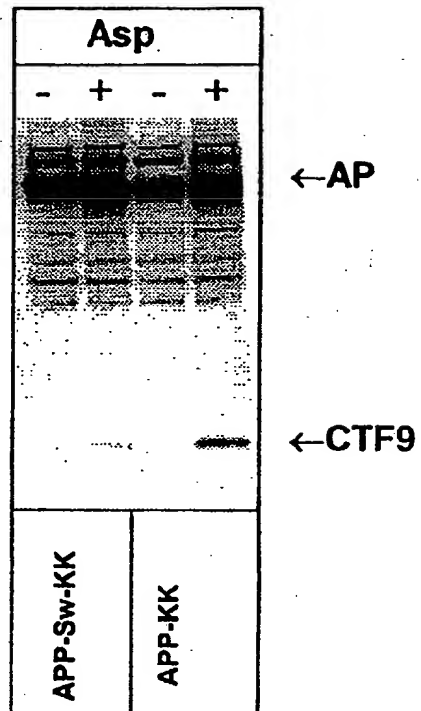


FIGURE 12

MAQALPWLLLMGAGVLP AHGTQH GIRLPLRSGLG GAPIGLRLPRETDEE  
PEEPGRRGSFVEMVDNLRGKSGQGYVEMTVGSP PQTLN ILVD TGSSNFA  
VGAAPHPFLHRYYQRLSS TYRDLRKGVYVPYTQ GKWEGELGTDLVSI PH  
GPNVTVRANIAAITESDKFFINGSNWEGILGLAYAEIARPDDSLEPPFDS  
LVKQTHVPNLFSLQLCGAGFPLNQSEVLASVGGSMIIGGIDHSLYTGSLW  
YTPIRREWYYEVIIVRVEINGQDLKMDCKEYNYDKSIVDSGTTNLR LPKK  
VFEAAVKS IKAASSTEKFPDGFWLGEQLVCWQAGTTPWNIFFVISLYLMG  
EVTNQSFRTITLPQOYLRFVEDVATSQDDCYKFAISQSSTGTVMGAVIME  
GFYVVFDRARKRIGFAVSACHVHDEFRTAAVEGPFVTLDMEDCGYNI PQT  
DESHHHHHH

- 2 -

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aaaa
1804

```

&lt;210&gt; 2

&lt;211&gt; 518

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 2

```

Met Gly Ala Leu Ala Arg Ala Leu Leu Leu Pro Leu Leu Ala Gln Trp
  1           5           10           15

Leu Leu Arg Ala Ala Pro Glu Leu Ala Pro Ala Pro Phe Thr Leu Pro
 20           25           30

Leu Arg Val Ala Ala Ala Thr Asn Arg Val Val Ala Pro Thr Pro Gly
 35           40           45

Pro Gly Thr Pro Ala Glu Arg His Ala Asp Gly Leu Ala Leu Ala Leu
 50           55           60

Glu Pro Ala Leu Ala Ser Pro Ala Gly Ala Ala Asn Phe Leu Ala Met
 65           70           75           80

Val Asp Asn Leu Gln Gly Asp Ser Gly Arg Gly Tyr Tyr Leu Glu Met
 85           90           95

Leu Ile Gly Thr Pro Pro Gln Lys Leu Gln Ile Leu Val Asp Thr Gly
100          105          110

Ser Ser Asn Phe Ala Val Ala Gly Thr Pro His Ser Tyr Ile Asp Thr
115          120          125

Tyr Phe Asp Thr Glu Arg Ser Ser Thr Tyr Arg Ser Lys Gly Phe Asp
130          135          140

Val Thr Val Lys Tyr Thr Gln Gly Ser Trp Thr Gly Phe Val Gly Glu
145          150          155          160

Asp Leu Val Thr Ile Pro Lys Gly Phe Asn Thr Ser Phe Leu Val Asn
165          170          175

Ile Ala Thr Ile Phe Glu Ser Glu Asn Phe Phe Leu Pro Gly Ile Lys
180          185          190

Trp Asn Gly Ile Leu Gly Leu Ala Tyr Ala Thr Leu Ala Lys Pro Ser
195          200          205

Ser Ser Leu Glu Thr Phe Phe Asp Ser Leu Val Thr Gln Ala Asn Ile
210          215          220

Pro Asn Val Phe Ser Met Gln Met Cys Gly Ala Gly Leu Pro Val Ala
225          230          235          240

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- 4 -

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2070

```

&lt;210&gt; 4

&lt;211&gt; 501

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 4

Met Ala Gln Ala Leu Pro Trp Leu Leu Trp Met Gly Ala Gly Val  
1 5 10 15

Leu Pro Ala His Gly Thr Gln His Gly Ile Arg Leu Pro Leu Arg Ser  
20 25 30

Gly Leu Gly Gly Ala Pro Leu Gly Leu Arg Leu Pro Arg Glu Thr Asp  
35 40 45

Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val  
50 55 60

Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr  
65 70 75 80

Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser  
85 90 95

Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr  
100 105 110



- 6 -

Gln Thr Asp Glu Ser Thr Leu Met Thr Ile Ala Tyr Val Met Ala Ala  
450 455 460

Ile Cys Ala Leu Phe Met Leu Pro Leu Cys Leu Met Val Cys Gln Trp  
465 470 475 480

Arg Cys Leu Arg Cys Leu Arg Gln Gln His Asp Asp Phe Ala Asp Asp  
485 490 495

Ile Ser Leu Leu Lys  
500

<210> 5  
<211> 1977  
<212> DNA  
<213> Homo sapiens

<400> 5  
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ccctgctggc caaagtcagt aggagaggat gcacagtttg ctatttgctt tagagacagg 1920  
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<210> 6  
<211> 476  
<212> PRT  
<213> Homo sapiens

<400> 6  
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- 8 -

Asp Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val  
 355 360 365

Met Gly Ala Val Ile Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala  
 370 375 380

Arg Lys Arg Ile Gly Phe Ala Val Ser Ala Cys His Val His Asp Glu  
 385 390 395 400

Phe Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu  
 405 410 415

Asp Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser Thr Leu Met Thr  
 420 425 430

Ile Ala Tyr Val Met Ala Ala Ile Cys Ala Leu Phe Met Leu Pro Leu  
 435 440 445

Cys Leu Met Val Cys Gln Trp Arg Cys Leu Arg Cys Leu Arg Gln Gln  
 450 455 460

His Asp Asp Phe Ala Asp Asp Ile Ser Leu Leu Lys  
 465 470 475

&lt;210&gt; 7

&lt;211&gt; 2043

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;400&gt; 7

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```

- 10 -

Asp Leu Lys Met Asp Cys Lys Glu Tyr Asn Tyr Asp Lys Ser Ile Val  
 275 280 285  
 Asp Ser Gly Thr Thr Asn Leu Arg Leu Pro Lys Lys Val Phe Glu Ala  
 290 295 300  
 Ala Val Lys Ser Ile Lys Ala Ala Ser Ser Thr Glu Lys Phe Pro Asp  
 305 310 315 320  
 Gly Phe Trp Leu Gly Glu Gln Leu Val Cys Trp Gln Ala Gly Thr Thr  
 325 330 335  
 Pro Trp Asn Ile Phe Pro Val Ile Ser Leu Tyr Leu Met Gly Glu Val  
 340 345 350  
 Thr Asn Gln Ser Phe Arg Ile Thr Ile Leu Pro Gln Gln Tyr Leu Arg  
 355 360 365  
 Pro Val Glu Asp Val Ala Thr Ser Gln Asp Asp Cys Tyr Lys Phe Ala  
 370 375 380  
 Val Ser Gln Ser Ser Thr Gly Thr Val Met Gly Ala Val Ile Met Glu  
 385 390 395 400  
 Gly Phe Tyr Val Val Phe Asp Arg Ala Arg Lys Arg Ile Gly Phe Ala  
 405 410 415  
 Val Ser Ala Cys His Val His Asp Glu Phe Arg Thr Ala Ala Val Glu  
 420 425 430  
 Gly Pro Phe Val Thr Ala Asp Met Glu Asp Cys Gly Tyr Asn Ile Pro  
 435 440 445  
 Gln Thr Asp Glu Ser Thr Leu Met Thr Ile Ala Tyr Val Met Ala Ala  
 450 455 460  
 Ile Cys Ala Leu Phe Met Leu Pro Leu Cys Leu Met Val Cys Gln Trp  
 465 470 475 480  
 Arg Cys Leu Arg Cys Leu Arg His Gln His Asp Asp Phe Ala Asp Asp  
 485 490 495  
 Ile Ser Leu Leu Lys  
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&lt;210&gt; 9

&lt;211&gt; 2088

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 9

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- 12 -

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile  
 165 170 175  
 Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu  
 180 185 190  
 Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val  
 195 200 205  
 Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys  
 210 215 220  
 Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu  
 225 230 235 240  
 Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu  
 245 250 255  
 Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile  
 260 265 270  
 Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg  
 275 280 285  
 Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu  
 290 295 300  
 Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys  
 305 310 315 320  
 Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg  
 325 330 335  
 Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp  
 340 345 350  
 Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu  
 355 360 365  
 Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala  
 370 375 380  
 Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn  
 385 390 395 400  
 Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe  
 405 410 415  
 Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His  
 420 425 430  
 Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala  
 435 440 445  
 Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu  
 450 455 460  
 Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala  
 465 470 475 480  
 Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn  
 485 490 495

- 14 -

```

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```

&lt;210&gt; 12

&lt;211&gt; 695

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 12

```

Met Leu Pro Gly Leu Ala Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1             5             10             15

```

```

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
      20             25             30

```

```

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
      35             40             45

```

```

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
      50             55             60

```

```

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
      65             70             75             80

```

```

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
      85             90             95

```

```

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
      100            105            110

```

```

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
      115            120            125

```

```

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
      130            135            140

```

```

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
      145            150            155            160

```

```

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
      165            170            175

```

```

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
      180            185            190

```

- 16 -

Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr  
 515 520 525  
 Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln  
 530 535 540  
 Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn  
 545 550 555 560  
 Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr  
 565 570 575  
 Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser  
 580 585 590  
 Glu Val Asn Leu Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val  
 595 600 605  
 His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys  
 610 615 620  
 Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val  
 625 630 635 640  
 Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile  
 645 650 655  
 His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg  
 660 665 670  
 His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys  
 675 680 685  
 Phe Phe Glu Gln Met Gln Asn  
 690 695

<210> 13  
 <211> 2088  
 <212> DNA  
 <213> Homo sapiens

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- 18 -

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys  
 210 215 220  
 Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu  
 225 230 235 240  
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 245 250 255  
 Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile  
 260 265 270  
 Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg  
 275 280 285  
 Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu  
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 Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys  
 305 310 315 320  
 Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg  
 325 330 335  
 Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp  
 340 345 350  
 Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu  
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 370 375 380  
 Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn  
 385 390 395 400  
 Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe  
 405 410 415  
 Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His  
 420 425 430  
 Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala  
 435 440 445  
 Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu  
 450 455 460  
 Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala  
 465 470 475 480  
 Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn  
 485 490 495  
 Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser  
 500 505 510  
 Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr  
 515 520 525  
 Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln  
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- 20 -

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&lt;210&gt; 16

&lt;211&gt; 697

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 16

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Met Leu Pro Gly Leu Ala Leu Leu Leu Ala Ala Trp Thr Ala Arg
 1             5             10             15

Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
      20             25             30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
      35             40             45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
      50             55             60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
      65             70             75             80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn
      85             90             95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val
      100            105            110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu
      115            120            125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys
      130            135            140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu
      145            150            155            160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile
      165            170            175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu
      180            185            190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val
      195            200            205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys
      210            215            220

Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu
      225            230            235            240

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- 22 -

Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser  
580 585 590

Glu Val Lys Met Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val  
595 600 605

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly Ser Asn Lys  
610 615 620

Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile Ala Thr Val  
625 630 635 640

Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile  
645 650 655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg  
660 665 670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys  
675 680 685

Phe Phe Glu Gln Met Gln Asn Lys Lys  
690 695

&lt;210&gt; 17

&lt;211&gt; 2094

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 17

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- 24 -

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg  
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 Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu  
 290 295 300  
 Glu Thr Pro Gly Asp Glu Asn Glu His Ala His Phe Gln Lys Ala Lys  
 305 310 315 320  
 Glu Arg Leu Glu Ala Lys His Arg Glu Arg Met Ser Gln Val Met Arg  
 325 330 335  
 Glu Trp Glu Glu Ala Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp  
 340 345 350  
 Lys Lys Ala Val Ile Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu  
 355 360 365  
 Gln Glu Ala Ala Asn Glu Arg Gln Gln Leu Val Glu Thr His Met Ala  
 370 375 380  
 Arg Val Glu Ala Met Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn  
 385 390 395 400  
 Tyr Ile Thr Ala Leu Gln Ala Val Pro Pro Arg Pro Arg His Val Phe  
 405 410 415  
 Asn Met Leu Lys Lys Tyr Val Arg Ala Glu Gln Lys Asp Arg Gln His  
 420 425 430  
 Thr Leu Lys His Phe Glu His Val Arg Met Val Asp Pro Lys Lys Ala  
 435 440 445  
 Ala Gln Ile Arg Ser Gln Val Met Thr His Leu Arg Val Ile Tyr Glu  
 450 455 460  
 Arg Met Asn Gln Ser Leu Ser Leu Leu Tyr Asn Val Pro Ala Val Ala  
 465 470 475 480  
 Glu Glu Ile Gln Asp Glu Val Asp Glu Leu Leu Gln Lys Glu Gln Asn  
 485 490 495  
 Tyr Ser Asp Asp Val Leu Ala Asn Met Ile Ser Glu Pro Arg Ile Ser  
 500 505 510  
 Tyr Gly Asn Asp Ala Leu Met Pro Ser Leu Thr Glu Thr Lys Thr Thr  
 515 520 525  
 Val Glu Leu Leu Pro Val Asn Gly Glu Phe Ser Leu Asp Asp Leu Gln  
 530 535 540  
 Pro Trp His Ser Phe Gly Ala Asp Ser Val Pro Ala Asn Thr Glu Asn  
 545 550 555 560  
 Glu Val Glu Pro Val Asp Ala Arg Pro Ala Ala Asp Arg Gly Leu Thr  
 565 570 575  
 Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser  
 580 585 590  
 Glu Val Asn Leu Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val  
 595 600 605

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&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 20

Met Leu Pro Gly Leu Ala Leu Leu Leu Leu Ala Ala Trp Thr Ala Arg  
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Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro  
 20 25 30

Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln  
 35 40 45

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp  
 50 55 60

Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu  
 65 70 75 80

Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn  
 85 90 95

Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val  
 100 105 110

Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu  
 115 120 125

Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys  
 130 135 140

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu  
 145 150 155 160

Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile  
 165 170 175

Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu  
 180 185 190

Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val  
 195 200 205

Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys  
 210 215 220

Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu  
 225 230 235 240

Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu  
 245 250 255

Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile  
 260 265 270

Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg  
 275 280 285

Val Pro Thr Thr Ala Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu  
 290 295 300

- 28 -

Ile Phe Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile  
645 650 655

His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg  
660 665 670

His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys  
675 680 685

Phe Phe Glu Gln Met Gln Asn Lys Lys  
690 695

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<211> 1341  
<212> DNA  
<213> Homo sapiens

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<210> 22  
<211> 446  
<212> PRT  
<213> Homo sapiens

<400> 22  
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His Gly Ile Arg Leu Pro Leu Arg Ser Gly Leu Gly Gly Ala Pro Leu  
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Gly Leu Arg Leu Pro Arg Glu Thr Asp Glu Glu Pro Glu Glu Pro Gly  
35 40 45

Arg Arg Gly Ser Phe Val Glu Met Val Asp Asn Leu Arg Gly Lys Ser  
50 55 60

- 30 -

Arg Ala Arg Lys Arg Ile Gly Phe Ala Val Ser Ala Cys His Val His  
405 410 415

Asp Glu Phe Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp  
420 425 430

Met Glu Asp Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser  
435 440 445

&lt;210&gt; 23

&lt;211&gt; 1380

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 23

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&lt;210&gt; 24

&lt;211&gt; 459

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 24

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Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Gly Ser Met Thr
  1           5           10          15

Ile Ser Asp Ser Pro Arg Glu Gln Asp Gly Ser Thr Gln His Gly Ile
      20           25           30

Arg Leu Pro Leu Arg Ser Gly Leu Gly Gly Ala Pro Leu Gly Leu Arg
      35           40           45

Leu Pro Arg Glu Thr Asp Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly
      50           55           60

Ser Phe Val Glu Met Val Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly
      65           70           75           80

Tyr Tyr Val Glu Met Thr Val Gly Ser Pro Pro Gln Thr Leu Asn Ile
      85           90           95

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- 32 -

Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu Asp  
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Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser  
 450 455

<210> 25  
 <211> 1302  
 <212> DNA  
 <213> Homo sapiens

<400> 25  
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 gtggagatgg tggacaacct gaggggcaag tcggggcagg gctactacgt ggagatgacc 180  
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<210> 26  
 <211> 433  
 <212> PRT  
 <213> Homo sapiens

<400> 26  
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 Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val Asp Asn Leu Arg  
 35 40 45  
 Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr Val Gly Ser Pro  
 50 55 60  
 Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser Ser Asn Phe Ala  
 65 70 75 80  
 Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr Tyr Gln Arg Gln  
 85 90 95  
 Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys Gly Val Tyr Val Pro Tyr  
 100 105 110

- 34 -

<210> 27  
 <211> 1278  
 <212> DNA  
 <213> Homo sapiens

<400> 27  
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 ggcaagtcgg ggcagggcta ctacgtggag atgaccgtgg gcagccccc gcagacgtc 180  
 aacatcctgg tggatacagg cagcagtaac ttgacgtgg gtgctgcccc ccacccttc 240  
 ctgcatcgt actaccagag gcagctgtcc agcacatacc gggacctccg gaaggggtg 300  
 tatgtccct acaccaggg caagtgggaa ggggagctgg gcaccgacct ggtaagcatc 360  
 cccctggcc ccaacgtcac tgtgcgtgcc aacattgctg ccatcactga atcagacaag 420  
 ttcttcacga acggctcga ctgggaaggc atcctggggc tggcctatgc tgagattgcc 480  
 aggcctgacg actccctgga gcctttcttt gactctctgg taaagcagac ccacgttccc 540  
 aacctcttct cctgcacct ttgtggtgct ggcttcccc tcaaccagtc tgaagtgtg 600  
 gcctctgtcg gaggagcat gatcattgga ggtatcgacc actcgtgtga cacaggcagt 660  
 ctctggtata caccatccg gcgggagtg tattatgagg tcatcattgt gcgggtggag 720  
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 gacagtggca ccaccaacct tcgtttgccc aagaaagtgt ttgaagctgc agtcaaattc 840  
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 gtgtgctggc aagcaggcac cacccttgg aacattttcc cagtcatttc actctaccta 960  
 atgggtgagg ttaccaacca gtcttccgc atcaccatcc ttccgcagca atacctgagg 1020  
 ccagtggag atgtggccac gtccaagac gactgttaca agtttgccat ctcacagtca 1080  
 tccacgggca ctgttatgg agctgttacc atggagggtc tctacgttgt ctttgatcgg 1140  
 gcccgaaaac gaattgctt tgctgtcagc gcttgccatg tgcacgatga gttcaggacg 1200  
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 cagacagatg agtcatga 1278

<210> 28  
 <211> 425  
 <212> PRT  
 <213> Homo sapiens

<400> 28  
 Met Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Gly Ser Met Thr  
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 Ile Ser Asp Ser Pro Leu Asp Ser Gly Ile Glu Thr Asp Gly Ser Phe  
 20 25 30  
 Val Glu Met Val Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr  
 35 40 45  
 Val Glu Met Thr Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val  
 50 55 60  
 Asp Thr Gly Ser Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe  
 65 70 75 80  
 Leu His Arg Tyr Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu  
 85 90 95  
 Arg Lys Gly Val Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu  
 100 105 110  
 Leu Gly Thr Asp Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val  
 115 120 125  
 Arg Ala Asn Ile Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn  
 130 135 140

- 36 -

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ctgcggctgc cccgggagac cgacgaagag cccgaggagc ccggccggag gggcagcttt 180
gtggagatgg tggacaacct gaggggcaag tcggggcagg gctactacgt ggagatgacc 240
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&lt;210&gt; 30

&lt;211&gt; 453

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 30

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Met Ala Gln Ala Leu Pro Trp Leu Leu Leu Trp Met Gly Ala Gly Val
  1              5              10              15

Leu Pro Ala His Gly Thr Gln His Gly Ile Arg Leu Pro Leu Arg Ser
      20              25              30

Gly Leu Gly Gly Ala Pro Leu Gly Leu Arg Leu Pro Arg Glu Thr Asp
      35              40              45

Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val
      50              55              60

Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr
      65              70              75              80

Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser
      85              90              95

Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr
      100             105             110

Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys Gly Val
      115             120             125

Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu Leu Gly Thr Asp
      130             135             140

Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val Arg Ala Asn Ile
      145             150             155             160

Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn Gly Ser Asn Trp
      165             170             175

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ggcaccacgc acggcatccg gctgcccctg cgcagcggcc tggggggcgc ccccttggg 120
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&lt;210&gt; 32

&lt;211&gt; 459

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 32

```

Met Ala Gln Ala Leu Pro Trp Leu Leu Leu Trp Met Gly Ala Gly Val
  1             5             10             15

Leu Pro Ala His Gly Thr Gln His Gly Ile Arg Leu Pro Leu Arg Ser
          20             25             30

Gly Leu Gly Gly Ala Pro Leu Gly Leu Arg Leu Pro Arg Glu Thr Asp
          35             40             45

Glu Glu Pro Glu Glu Pro Gly Arg Arg Gly Ser Phe Val Glu Met Val
          50             55             60

Asp Asn Leu Arg Gly Lys Ser Gly Gln Gly Tyr Tyr Val Glu Met Thr
          65             70             75             80

Val Gly Ser Pro Pro Gln Thr Leu Asn Ile Leu Val Asp Thr Gly Ser
          85             90             95

Ser Asn Phe Ala Val Gly Ala Ala Pro His Pro Phe Leu His Arg Tyr
          100            105            110

Tyr Gln Arg Gln Leu Ser Ser Thr Tyr Arg Asp Leu Arg Lys Gly Val
          115            120            125

Tyr Val Pro Tyr Thr Gln Gly Lys Trp Glu Gly Glu Leu Gly Thr Asp
          130            135            140

Leu Val Ser Ile Pro His Gly Pro Asn Val Thr Val Arg Ala Asn Ile
          145            150            155            160

Ala Ala Ile Thr Glu Ser Asp Lys Phe Phe Ile Asn Gly Ser Asn Trp
          165            170            175

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- 40 -

Ser Glu Gln Gln Arg Arg Pro Arg Asp Pro Glu Val Val Asn Asp Glu  
 1 5 10 15

Ser Ser Leu Val Arg His Arg Trp Lys  
 20 25

<210> 34  
 <211> 19  
 <212> PRT  
 <213> Homo sapiens

<400> 34  
 Ser Glu Gln Leu Arg Gln Gln His Asp Asp Phe Ala Asp Asp Ile Ser  
 1 5 10 15

Leu Leu Lys

<210> 35  
 <211> 29  
 <212> DNA  
 <213> Homo sapiens

<400> 35  
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<210> 36  
 <211> 36  
 <212> DNA  
 <213> Homo sapiens

<400> 36  
 gaaagctttc atgactcatc tgtctgtgga atgttg 36

<210> 37  
 <211> 39  
 <212> DNA  
 <213> Homo sapiens

<400> 37  
 gatcgatgac tatctctgac tctccgcgtg aacaggacg 39

<210> 38  
 <211> 39  
 <212> DNA  
 <213> Homo sapiens

<400> 38  
 gatccgtcct gttcacgcgg agagtcagag atagtcac 39

<210> 39  
 <211> 77  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence: Hu-Asp2

<400> 39  
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 ccgggagacc gacgaag 77

- 42 -

&lt;210&gt; 46

&lt;211&gt; 24

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: 6-His tag

&lt;400&gt; 46

gatccatggt gatggtgatg atgc

24

&lt;210&gt; 47

&lt;211&gt; 22

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer

&lt;400&gt; 47

gactgaccac tcgaccaggt tc

22

&lt;210&gt; 48

&lt;211&gt; 51

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer

&lt;400&gt; 48

cgaattaaat tccagcacac tggctacttc ttgttctgca tctcaaagaa c

51

&lt;210&gt; 49

&lt;211&gt; 26

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer

&lt;400&gt; 49

cgaattaaat tccagcacac tggcta

26

&lt;210&gt; 50

&lt;211&gt; 1287

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Hu-Asp2(b)  
delta TM

&lt;400&gt; 50

atggcccag cctgcccctg gctcctgctg tggatgggag cgggagtgt gcctgcccac 60  
 ggcaaccagc acggcatccg gctgcccctg cgcagcggcc tggggggcgc cccctgggg 120  
 ctgcccgtgc cccgggagac cgaagaagag cccgaggagc ccggccggag gggcagcttt 180  
 gtggagatgg tggacaacct gaggggcaag tgggggcagg gctactacgt ggagatgacc 240  
 gtgggcagcc cccgcagac gctcaacatc ctggtggata caggcagcag taactttgca 300  
 gtgggtgctg ccccccaccc ctctctgcat cgctactacc agaggcagct gtccagcaca 360  
 taccgggacc tccggaaggg tgtgtatgtg cctacaccc agggcaagt ggaaggggag 420  
 ctgggcaccg acctggttaag catcccccat ggcccacag tcactgtgcg tgccaacatt 480  
 gctgccatca ctgaatcaga caagttcttc atcaacggct ccaactggga aggcacacct 540

- 44 -

Ser Met Ile Ile Gly Gly Ile Asp His Ser Leu Tyr Thr Gly Ser Leu  
 210 215 220  
 Trp Tyr Thr Pro Ile Arg Arg Glu Trp Tyr Tyr Glu Val Ile Ile Val  
 225 230 235 240  
 Arg Val Glu Ile Asn Gly Gln Asp Leu Lys Met Asp Cys Lys Glu Tyr  
 245 250 255  
 Asn Tyr Asp Lys Ser Ile Val Asp Ser Gly Thr Thr Asn Leu Arg Leu  
 260 265 270  
 Pro Lys Lys Val Phe Glu Ala Ala Val Lys Ser Ile Lys Ala Ala Ser  
 275 280 285  
 Ser Thr Glu Lys Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val  
 290 295 300  
 Cys Trp Gln Ala Gly Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser  
 305 310 315 320  
 Leu Tyr Leu Met Gly Glu Val Thr Asn Gln Ser Phe Arg Ile Thr Ile  
 325 330 335  
 Leu Pro Gln Gln Tyr Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln  
 340 345 350  
 Asp Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val  
 355 360 365  
 Met Gly Ala Val Ile Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala  
 370 375 380  
 Arg Lys Arg Ile Gly Phe Ala Val Ser Ala Cys His Val His Asp Glu  
 385 390 395 400  
 Phe Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu  
 405 410 415  
 Asp Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser  
 420 425

&lt;210&gt; 52

&lt;211&gt; 1305

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

 <223> Description of Artificial Sequence: Hu-Asp2(b)  
 delta TM

&lt;400&gt; 52

atggcccaag cctgcccctg gctcctgctg tggatgggag cgggagtgct gcctgcccac 60  
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 gtgggcagcc ccccgagac gctcaacatc ctggtggata caggcagcag taactttgca 300  
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 gggctggcct atgctgagat tgccaggctt tgtggtgctg gcttccccct caaccagtct 600

- 46 -

Ser Met Ile Ile Gly Gly Ile Asp His Ser Leu Tyr Thr Gly Ser Leu  
 210 215 220  
 Trp Tyr Thr Pro Ile Arg Arg Glu Trp Tyr Tyr Glu Val Ile Ile Val  
 225 230 235 240  
 Arg Val Glu Ile Asn Gly Gln Asp Leu Lys Met Asp Cys Lys Glu Tyr  
 245 250 255  
 Asn Tyr Asp Lys Ser Ile Val Asp Ser Gly Thr Thr Asn Leu Arg Leu  
 260 265 270  
 Pro Lys Lys Val Phe Glu Ala Ala Val Lys Ser Ile Lys Ala Ala Ser  
 275 280 285  
 Ser Thr Glu Lys Phe Pro Asp Gly Phe Trp Leu Gly Glu Gln Leu Val  
 290 295 300  
 Cys Trp Gln Ala Gly Thr Thr Pro Trp Asn Ile Phe Pro Val Ile Ser  
 305 310 315 320  
 Leu Tyr Leu Met Gly Glu Val Thr Asn Gln Ser Phe Arg Ile Thr Ile  
 325 330 335  
 Leu Pro Gln Gln Tyr Leu Arg Pro Val Glu Asp Val Ala Thr Ser Gln  
 340 345 350  
 Asp Asp Cys Tyr Lys Phe Ala Ile Ser Gln Ser Ser Thr Gly Thr Val  
 355 360 365  
 Met Gly Ala Val Ile Met Glu Gly Phe Tyr Val Val Phe Asp Arg Ala  
 370 375 380  
 Arg Lys Arg Ile Gly Phe Ala Val Ser Ala Cys His Val His Asp Glu  
 385 390 395 400  
 Phe Arg Thr Ala Ala Val Glu Gly Pro Phe Val Thr Leu Asp Met Glu  
 405 410 415  
 Asp Cys Gly Tyr Asn Ile Pro Gln Thr Asp Glu Ser His His His His  
 420 425 430  
 His His

&lt;210&gt; 54

&lt;211&gt; 2310

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 54

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 ctgaacatgc acatgaatgt ccagaatggg aagtgggatt cagatccatc agggaccaa 180  
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- 48 -

Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu  
 145 150 155 160  
 Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile  
 165 170 175  
 Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu  
 180 185 190  
 Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val  
 195 200 205  
 Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys  
 210 215 220  
 Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu  
 225 230 235 240  
 Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu  
 245 250 255  
 Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile  
 260 265 270  
 Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg  
 275 280 285  
 Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile  
 290 295 300  
 Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe  
 305 310 315 320  
 Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr  
 325 330 335  
 Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr  
 340 345 350  
 Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala  
 355 360 365  
 Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp  
 370 375 380  
 Glu Asn Glu His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala  
 385 390 395 400  
 Lys His Arg Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala  
 405 410 415  
 Glu Arg Gln Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile  
 420 425 430  
 Gln His Phe Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn  
 435 440 445  
 Glu Arg Gln Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met  
 450 455 460  
 Leu Asn Asp Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu  
 465 470 475 480

- 50 -

&lt;400&gt; 56

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atgattagtg aaccaaggat cagttacgga aacgatgtct tcatgccatc tttagccgaa 1740
acgaaaacca ccgtggagct ccttcccgtg aatggagagt ttagcctgga cgatctccag 1800
ccgtggcatt cttttggggc tgactctgtg ccagccaaca cagaaaacga agttgagcct 1860
gttgatgccc gccctgctgc cgaccgagga ctgaccactc gaccaggttc tgggttgaca 1920
aatatcaaga cggaggagat ctctgaagtg aagatggatg cagaattccg acatgactca 1980
ggatatgaag ttcatacatc aaaattggtg ttctttgcag aagatgtggg ttcaaacaaa 2040
ggtgcaatca ttggactcat ggtgggcggg gttgtcatag cgacagttag cgtcatcacc 2100
ttggtgatgc tgaagaagaa acagtacaca tccattcatt atggtgtggg ggaggttgac 2160
gccgctgtca cccagagga gcgccacctg tccaagatgc agcagaacgg ctacgaaaat 2220
ccaacctaca agttctttga gcagatgcag aac 2253

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&lt;210&gt; 57

&lt;211&gt; 751

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 57

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Met Leu Pro Gly Leu Ala Leu Leu Leu Ala Ala Trp Thr Ala Arg
  1             5             10            15
Ala Leu Glu Val Pro Thr Asp Gly Asn Ala Gly Leu Leu Ala Glu Pro
          20             25             30
Gln Ile Ala Met Phe Cys Gly Arg Leu Asn Met His Met Asn Val Gln
          35             40             45
Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp
          50             55             60
Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu
          65             70             75             80

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- 52 -

Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn Glu Arg Gln  
 420 425 430  
 Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met Leu Asn Asp  
 435 440 445  
 Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu Gln Ala Val  
 450 455 460  
 Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys Tyr Val Arg  
 465 470 475 480  
 Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe Glu His Val  
 485 490 495  
 Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser Gln Val Met  
 500 505 510  
 Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser Leu Ser Leu  
 515 520 525  
 Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp Glu Val Asp  
 530 535 540  
 Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val Leu Ala Asn  
 545 550 555 560  
 Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala Leu Met Pro  
 565 570 575  
 Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro Val Asn Gly  
 580 585 590  
 Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe Gly Ala Asp  
 595 600 605  
 Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val Asp Ala Arg  
 610 615 620  
 Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser Gly Leu Thr  
 625 630 635 640  
 Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp Ala Glu Phe  
 645 650 655  
 Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys Leu Val Phe Phe  
 660 665 670  
 Ala Glu Asp Val Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val  
 675 680 685  
 Gly Gly Val Val Ile Ala Thr Val Ile Val Ile Thr Leu Val Met Leu  
 690 695 700  
 Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val Glu Val Asp  
 705 710 715 720  
 Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn  
 725 730 735  
 Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn  
 740 745 750



- 54 -

Asn Gly Lys Trp Asp Ser Asp Pro Ser Gly Thr Lys Thr Cys Ile Asp  
 50 55 60  
 Thr Lys Glu Gly Ile Leu Gln Tyr Cys Gln Glu Val Tyr Pro Glu Leu  
 65 70 75 80  
 Gln Ile Thr Asn Val Val Glu Ala Asn Gln Pro Val Thr Ile Gln Asn  
 85 90 95  
 Trp Cys Lys Arg Gly Arg Lys Gln Cys Lys Thr His Pro His Phe Val  
 100 105 110  
 Ile Pro Tyr Arg Cys Leu Val Gly Glu Phe Val Ser Asp Ala Leu Leu  
 115 120 125  
 Val Pro Asp Lys Cys Lys Phe Leu His Gln Glu Arg Met Asp Val Cys  
 130 135 140  
 Glu Thr His Leu His Trp His Thr Val Ala Lys Glu Thr Cys Ser Glu  
 145 150 155 160  
 Lys Ser Thr Asn Leu His Asp Tyr Gly Met Leu Leu Pro Cys Gly Ile  
 165 170 175  
 Asp Lys Phe Arg Gly Val Glu Phe Val Cys Cys Pro Leu Ala Glu Glu  
 180 185 190  
 Ser Asp Asn Val Asp Ser Ala Asp Ala Glu Glu Asp Asp Ser Asp Val  
 195 200 205  
 Trp Trp Gly Gly Ala Asp Thr Asp Tyr Ala Asp Gly Ser Glu Asp Lys  
 210 215 220  
 Val Val Glu Val Ala Glu Glu Glu Glu Val Ala Glu Val Glu Glu Glu  
 225 230 235 240  
 Glu Ala Asp Asp Asp Glu Asp Asp Glu Asp Gly Asp Glu Val Glu Glu  
 245 250 255  
 Glu Ala Glu Glu Pro Tyr Glu Glu Ala Thr Glu Arg Thr Thr Ser Ile  
 260 265 270  
 Ala Thr Thr Thr Thr Thr Thr Thr Glu Ser Val Glu Glu Val Val Arg  
 275 280 285  
 Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile  
 290 295 300  
 Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe  
 305 310 315 320  
 Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr  
 325 330 335  
 Cys Met Ala Val Cys Gly Ser Ala Met Ser Gln Ser Leu Leu Lys Thr  
 340 345 350  
 Thr Gln Glu Pro Leu Ala Arg Asp Pro Val Lys Leu Pro Thr Thr Ala  
 355 360 365  
 Ala Ser Thr Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp  
 370 375 380

- 56 -

Val Met Leu Lys Lys Lys Gln Tyr Thr Ser Ile His His Gly Val Val  
725 730 735

Glu Val Asp Ala Ala Val Thr Pro Glu Glu Arg His Leu Ser Lys Met  
740 745 750

Gln Gln Asn Gly Tyr Glu Asn Pro Thr Tyr Lys Phe Phe Glu Gln Met  
755 760 765

Gln Asn Lys Lys  
770

&lt;210&gt; 60

&lt;211&gt; 2259

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 60

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atgctgcccg gtttggcact gctcctgctg gccgcctgga cggctcgggc gctggaggta 60
cccactgatg gtaatgctgg cctgctggct gaaccccaga ttgccatgtt ctgtggcaga 120
ctgaacatgc acatgaatgt ccagaatggg aagtgggatt cagatccatc agggaccaa 180
acctgcattg ataccaagga aggcacccct cagtattgcc aagaagtcta ccctgaactg 240
cagatcacca atgtggtaga agccaacca ccagtgaaca tccagaactg gtgcaagcg 300
ggccgcaagc agtgcaagac ccatccccac tttgtgattc cctaccgctg cttagtgggt 360
gagtttgtaa gtgatgccct tctcgttcct gacaagtgc aattcttaca ccaggagagg 420
atggatgttt gcgaaactca tcttcaactg cacaccgtcg ccaaagagac atgcagtgg 480
aagagtacca acttgcataga ctacggcatg ttgctgccc tgcgaattga caagttccga 540
ggggttagagt ttgtgtgttg cccactggct gaagaaagtg acaatgtgga ttctgctgat 600
gctgaggagg atgactcggg tgtctggttg gccggagcag acacagacta tgcagatggg 660
agtgaagaca aagtagtaga agtagcagag gaggagaag tggtgaggt ggaagaagaa 720
gaagccgatg atgacgagga cgatgaggat ggtgatgagg tagaggaaga ggctgaggaa 780
ccctacgaag aagccacaga gagaaccacc agcattgcca ccaccaccac caccaccaca 840
gagtcctgtg aagaggtggg tgcagaggtg tgctctgaac aagccgagac ggggccgtgc 900
cgagcaatga tctcccgtg gtactttgat gtgactgaag ggaagtgtgc cccattcttt 960
tacggcggat gtggcggaac ccggaacaac tttgacacag aagagtactg catggccgtg 1020
tgtggcagcg ccattcctac aacagcagcc agtaccctg atgccgttga caagtatctc 1080
gagacacctg gggatgagaa tgaacatgcc cattccaga aagccaaaga gaggcttgag 1140
gccaaagcag gagagagaat gtcccaggct atgagagaat ggaagaggc agaacgtcaa 1200
gcaaaagaat tgcctaaagc tgataagaag gcagtattcc agcatttcca ggagaaagt 1260
gaatctttgg aacaggaagc agccaacgag agacagcagc tggtgagac acacatggcc 1320
agagtggagg ccatgctcaa tgaccgccc cgcctggccc tggagaacta catcaccgct 1380
ctgcaggctg tctctcctcg gcctcgctac gtgttcaata tgctaaagaa gtatgtccgc 1440
gcagaacaga aggacagaca gcacacccta aagcatttcg agcatgtgcg catgggtggat 1500
cccaagaaag ccgctcagat ccggtcccag gttatgacac acctccgtgt gatttatgag 1560
cgcatgaatc agtctctctc cctgctctac aacgtgcctg cagtggcga ggagattcag 1620
gatgaagtgt atgagctgct tcagaaagag caaaactatt cagatgacgt cttggccaac 1680
atgattagtg aaccaaggat cagttacgga aacgatgctc tcatgccac tttgaccgaa 1740
acgaaaacca ccgtggagct ccttcccgtg aatggagagt tcagcctgga cgatctccag 1800
ccgtggcatt cttttggggc tgactctgtg ccagccaaaca cagaaaacga agttgagcct 1860
gttgatgcc gccctgctgc cgaccgagga ctgaccactc gaccaggttc tgggttgaca 1920
aatatcaaga cggaggagat ctctgaagtg aagatggatg cagaattccg acatgactca 1980
ggatataga ttcacatca aaaattggtg ttctttgcag aagatgtggg ttcaaacaaa 2040
ggtgcaatca ttggaactcat ggtgggcggg tttgtcatag cgacagtgat cgtcatcacc 2100
ttggtgatgc tgaagaagaa acagtacaca tccattcatc atggtgtggg ggagggtgac 2160
gccgtgtgca cccagagga gcgccacctg tccaagatgc agcagaacgg ctacgaaaat 2220
ccaacctaca agttctttga gcagatgcag aacaagaag 2259

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&lt;210&gt; 61

&lt;211&gt; 753

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

- 58 -

Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr  
 325 330 335  
 Cys Met Ala Val Cys Gly Ser Ala Ile Pro Thr Thr Ala Ala Ser Thr  
 340 345 350  
 Pro Asp Ala Val Asp Lys Tyr Leu Glu Thr Pro Gly Asp Glu Asn Glu  
 355 360 365  
 His Ala His Phe Gln Lys Ala Lys Glu Arg Leu Glu Ala Lys His Arg  
 370 375 380  
 Glu Arg Met Ser Gln Val Met Arg Glu Trp Glu Glu Ala Glu Arg Gln  
 385 390 395 400  
 Ala Lys Asn Leu Pro Lys Ala Asp Lys Lys Ala Val Ile Gln His Phe  
 405 410 415  
 Gln Glu Lys Val Glu Ser Leu Glu Gln Glu Ala Ala Asn Glu Arg Gln  
 420 425 430  
 Gln Leu Val Glu Thr His Met Ala Arg Val Glu Ala Met Leu Asn Asp  
 435 440 445  
 Arg Arg Arg Leu Ala Leu Glu Asn Tyr Ile Thr Ala Leu Gln Ala Val  
 450 455 460  
 Pro Pro Arg Pro Arg His Val Phe Asn Met Leu Lys Lys Tyr Val Arg  
 465 470 475 480  
 Ala Glu Gln Lys Asp Arg Gln His Thr Leu Lys His Phe Glu His Val  
 485 490 495  
 Arg Met Val Asp Pro Lys Lys Ala Ala Gln Ile Arg Ser Gln Val Met  
 500 505 510  
 Thr His Leu Arg Val Ile Tyr Glu Arg Met Asn Gln Ser Leu Ser Leu  
 515 520 525  
 Leu Tyr Asn Val Pro Ala Val Ala Glu Glu Ile Gln Asp Glu Val Asp  
 530 535 540  
 Glu Leu Leu Gln Lys Glu Gln Asn Tyr Ser Asp Asp Val Leu Ala Asn  
 545 550 555 560  
 Met Ile Ser Glu Pro Arg Ile Ser Tyr Gly Asn Asp Ala Leu Met Pro  
 565 570 575  
 Ser Leu Thr Glu Thr Lys Thr Thr Val Glu Leu Leu Pro Val Asn Gly  
 580 585 590  
 Glu Phe Ser Leu Asp Asp Leu Gln Pro Trp His Ser Phe Gly Ala Asp  
 595 600 605  
 Ser Val Pro Ala Asn Thr Glu Asn Glu Val Glu Pro Val Asp Ala Arg  
 610 615 620  
 Pro Ala Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser Gly Leu Thr  
 625 630 635 640  
 Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Lys Met Asp Ala Glu Phe  
 645 650 655

- 60 -

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: synthetic

&lt;400&gt; 65

Arg Arg Gly Gly Val Val Ile Ala Thr Val Ile Val Gly Glu Arg  
 1 5 10 15

&lt;210&gt; 66

&lt;211&gt; 518

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 66

Met Gly Ala Leu Ala Arg Ala Leu Leu Leu Pro Leu Leu Ala Gln Trp  
 1 5 10 15

Leu Leu Arg Ala Ala Pro Glu Leu Ala Pro Ala Pro Phe Thr Leu Pro  
 20 25 30

Leu Arg Val Ala Ala Ala Thr Asn Arg Val Val Ala Pro Thr Pro Gly  
 35 40 45

Pro Gly Thr Pro Ala Glu Arg His Ala Asp Gly Leu Ala Leu Ala Leu  
 50 55 60

Glu Pro Ala Leu Ala Ser Pro Ala Gly Ala Ala Asn Phe Leu Ala Met  
 65 70 75 80

Val Asp Asn Leu Gln Gly Asp Ser Gly Arg Gly Tyr Tyr Leu Glu Met  
 85 90 95

Leu Ile Gly Thr Pro Pro Gln Lys Leu Gln Ile Leu Val Asp Thr Gly  
 100 105 110

Ser Ser Asn Phe Ala Val Ala Gly Thr Pro His Ser Tyr Ile Asp Thr  
 115 120 125

Tyr Phe Asp Thr Glu Arg Ser Ser Thr Tyr Arg Ser Lys Gly Phe Asp  
 130 135 140

Val Thr Val Lys Tyr Thr Gln Gly Ser Trp Thr Gly Phe Val Gly Glu  
 145 150 155 160

Asp Leu Val Thr Ile Pro Lys Gly Phe Asn Thr Ser Phe Leu Val Asn  
 165 170 175

Ile Ala Thr Ile Phe Glu Ser Glu Asn Phe Phe Leu Pro Gly Ile Lys  
 180 185 190

Trp Asn Gly Ile Leu Gly Leu Ala Tyr Ala Thr Leu Ala Lys Pro Ser  
 195 200 205

Ser Ser Leu Glu Thr Phe Phe Asp Ser Leu Val Thr Gln Ala Asn Ile  
 210 215 220

Pro Asn Val Phe Ser Met Gln Met Cys Gly Ala Gly Leu Pro Val Ala  
 225 230 235 240

- 62 -

Leu Leu Arg Ala Ala Pro Glu Leu Ala Pro Ala Pro Phe Thr Leu Pro  
 20 25 30  
 Leu Arg Val Ala Ala Ala Thr Asn Arg Val Val Ala Pro Thr Pro Gly  
 35 40 45  
 Pro Gly Thr Pro Ala Glu Arg His Ala Asp Gly Leu Ala Leu Ala Leu  
 50 55 60  
 Glu Pro Ala Leu Ala Ser Pro Ala Gly Ala Ala Asn Phe Leu Ala Met  
 65 70 75 80  
 Val Asp Asn Leu Gln Gly Asp Ser Gly Arg Gly Tyr Tyr Leu Glu Met  
 85 90 95  
 Leu Ile Gly Thr Pro Pro Gln Lys Leu Gln Ile Leu Val Asp Thr Gly  
 100 105 110  
 Ser Ser Asn Phe Ala Val Ala Gly Thr Pro His Ser Tyr Ile Asp Thr  
 115 120 125  
 Tyr Phe Asp Thr Glu Arg Ser Ser Thr Tyr Arg Ser Lys Gly Phe Asp  
 130 135 140  
 Val Thr Val Lys Tyr Thr Gln Gly Ser Trp Thr Gly Phe Val Gly Glu  
 145 150 155 160  
 Asp Leu Val Thr Ile Pro Lys Gly Phe Asn Thr Ser Phe Leu Val Asn  
 165 170 175  
 Ile Ala Thr Ile Phe Glu Ser Glu Asn Phe Phe Leu Pro Gly Ile Lys  
 180 185 190  
 Trp Asn Gly Ile Leu Gly Leu Ala Tyr Ala Thr Leu Ala Lys Pro Ser  
 195 200 205  
 Ser Ser Leu Glu Thr Phe Phe Asp Ser Leu Val Thr Gln Ala Asn Ile  
 210 215 220  
 Pro Asn Val Phe Ser Met Gln Met Cys Gly Ala Gly Leu Pro Val Ala  
 225 230 235 240  
 Gly Ser Gly Thr Asn Gly Gly Ser Leu Val Leu Gly Gly Ile Glu Pro  
 245 250 255  
 Ser Leu Tyr Lys Gly Asp Ile Trp Tyr Thr Pro Ile Lys Glu Glu Trp  
 260 265 270  
 Tyr Tyr Gln Ile Glu Ile Leu Lys Leu Glu Ile Gly Gly Gln Ser Leu  
 275 280 285  
 Asn Leu Asp Cys Arg Glu Tyr Asn Ala Asp Lys Ala Ile Val Asp Ser  
 290 295 300  
 Gly Thr Thr Leu Leu Arg Leu Pro Gln Lys Val Phe Asp Ala Val Val  
 305 310 315 320  
 Glu Ala Val Ala Arg Ala Ser Leu Ile Pro Glu Phe Ser Asp Gly Phe  
 325 330 335  
 Trp Thr Gly Ser Gln Leu Ala Cys Trp Thr Asn Ser Glu Thr Pro Trp  
 340 345 350

- 64 -

Val Ala Gly Ser Gly Thr Asn Gly Gly Ser Leu Val Leu Gly Gly Ile  
                   180                  185                  190  
 Glu Pro Ser Leu Tyr Lys Gly Asp Ile Trp Tyr Thr Pro Ile Lys Glu  
                   195                  200                  205  
 Glu Trp Tyr Tyr Gln Ile Glu Ile Leu Lys Leu Glu Ile Gly Gly Gln  
                   210                  215                  220  
 Ser Leu Asn Leu Asp Cys Arg Glu Tyr Asn Ala Asp Lys Ala Ile Val  
                   225                  230                  235                  240  
 Asp Ser Gly Thr Thr Leu Leu Arg Leu Pro Gln Lys Val Phe Asp Ala  
                   245                  250                  255  
 Val Val Glu Ala Val Ala Arg Ala Ser Leu Ile Pro Glu Phe Ser Asp  
                   260                  265                  270  
 Gly Phe Trp Thr Gly Ser Gln Leu Ala Cys Trp Thr Asn Ser Glu Thr  
                   275                  280                  285  
 Pro Trp Ser Tyr Phe Pro Lys Ile Ser Ile Tyr Leu Arg Asp Glu Asn  
                   290                  295                  300  
 Ser Ser Arg Ser Phe Arg Ile Thr Ile Leu Pro Gln Leu Tyr Ile Gln  
                   305                  310                  315                  320  
 Pro Met Met Gly Ala Gly Leu Asn Tyr Glu Cys Tyr Arg Phe Gly Ile  
                   325                  330                  335  
 Ser Pro Ser Thr Asn Ala Leu Val Ile Gly Ala Thr Val Met Glu Gly  
                   340                  345                  350  
 Phe Tyr Val Ile Phe Asp Arg Ala Gln Lys Arg Val Gly Phe Ala Ala  
                   355                  360                  365  
 Ser Pro Cys Ala Glu Ile Ala Gly Ala Ala Val Ser Glu Ile Ser Gly  
                   370                  375                  380  
 Pro Phe Ser Thr Glu Asp Val Ala Ser Asn Cys Val Pro Ala Gln Ser  
                   385                  390                  395                  400  
 Leu Ser Glu Pro Ile Leu Trp His His His His His His  
                   405                  410

&lt;210&gt; 69

&lt;211&gt; 8

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Peptide

&lt;400&gt; 69

Gly Leu Ala Leu Ala Leu Glu Pro

1

5

&lt;210&gt; 70

&lt;211&gt; 8

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

- 66 -

&lt;223&gt; Description of Artificial Sequence: Primer

&lt;400&gt; 75

cgctttctcg agctaattgt gatggtgatg gtgccacaaa atgggctcgc tcaaaga 57

&lt;210&gt; 76

&lt;211&gt; 15

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: Peptide

&lt;400&gt; 76

Arg Arg Gly Gly Val Val Ile Ala Thr Val Ile Val Gly Glu Arg  
1 5 10 15